Genetic Structure and Dynamics of All-hybrid Edible Frog Populations

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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aus

Dänemark

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Zürich 2010

Genetic structure and function of all-hybrid edible frog populations

by

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PhD thesis at
Ecology, Zoological Institute
University of Zurich, Switzerland
October 2009

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Summary

Hybridization is quantitatively important on an evolutionary scale and has several consequences. One consequence is the formation of new taxa. Most hybrid plant taxa are sexual species formed by tetraploidization, whereas many animal hybrid taxa have some form of clonal reproduction and are therefore considered evolutionary dead ends. The European hybrid edible frog, *Pelophylax esculentus*, is a biologically unique hybrid, because it forms three different breeding systems. In two of the breeding systems, the hybrids reproduce clonally with one or the other parental species. In contrast, the third breeding system consists of all-hybrid populations where the hybrids are 1) reproductively independent and 2) might have sexual reproduction with genetic recombination. These two points represent two important steps towards speciation.

P. esculentus is a hybrid between the pool frog, *P. lessonae* (genotype LL), and the marsh frog, *P. ridibundus* (genotype RR). In the all-hybrid populations, hybrids are either diploid, LR, or triploid, LLR or LRR. LR frogs make LR and/or R gametes, LLR frogs make L gametes and LRR frogs make R gametes. Frogs of different genotypes are thus dependent on mating with each other for reproduction. The present PhD thesis comprises four independent chapters, about all-hybrid populations, all aiming to increase our understanding of the structure and dynamics of all-hybrid populations of *P. esculentus*.

In chapter 1 it is demonstrated that all-hybrid populations of diploid (LR) and triploid (LLR and LRR) *P. esculentus* have indeed acquired sexual reproduction. This is of evolutionary importance for the hybrids, because clonal reproduction comes at the expense of genetic diversity and the ability to purge deleterious mutations. First, microsatellite marker analysis on parents and offspring from a crossing experiment showed that triploid hybrids of both sexes and genotypes (LLR and LRR) recombined their homospecific genomes. Second, the great majority of natural populations investigated had low multilocus linkage disequilibrium, indicating a high recombination rate. As predicted from mating system models, the L genome had constant, low levels of linkage disequilibrium, whereas linkage disequilibrium in the R genome showed a significant reduction with increasing proportion of recombining triploids. This direct evidence of sexual reproduction in *P. esculentus* calls for a change of the conventional view of hybridogens as clonally reproducing diploids. Rather, hybridogens can be independent sexually reproducing units with an evolutionary potential.

Chapter 2 provides data and interpretations on gamete types and sex determination that are essential for understanding the function and evolutionary potential of and the threats to

this intriguing system. Gamete patterns in hybrids are generally interesting, because triploid individuals often play a key role in speciation by hybridization. An understanding of the gamete types (ploidy and genomic content) and stability of hybrid populations with triploid individuals is therefore of importance for exploring the role of hybridization in evolution. Dissection of metamorphs from a crossing experiment confirmed that sex determination in the all-hybrid populations is an XX-XY system with the Y confined to the L genome. From microsatellite analysis of the crossed parents and their offspring, gamete frequencies could be deduced: Triploids of both sexes mostly made haploid gametes with the genome they had in double dose, however LLR females also made approximately 10% LL gametes by automixis. LR frogs showed much variation in their gamete production: In LRR-rich populations, their LR sperm production was sufficiently high (22%) to explain the observed proportion of LRR males, the formation of which has not previously been understood. This suggests that all-hybrid populations constitute not one, but several intrinsically different breeding systems. A model was constructed to calculate equilibrium genotype proportions for different population types on the basis of the gamete proportions found. These equilibria agreed well with empirical literature data. The model also predicted that tetraploidization could occur if the survival or fertility of both males and females increased. Whether introduction of hybrid or parental species individuals with new genetic variation would threaten the all-hybrid populations by promoting the survival of non-hybrids cannot be predicted without further knowledge about the mechanisms behind non-hybrid inviability. However, at least R genomes with Y factor are predicted to be invasive, if introduced, and would burden the all-hybrid populations with increased mortality from non-hybrid production.

Chapter 3 aims to asses the importance of selection in structuring all-hybrid *Pelophylax esculentus* populations. The role of differential selection in determining the geographic distribution of genotypes in hybrid systems has long been discussed, but not settled. With data from 12 Swedish ponds, it is first shown that - in spite of significant genotype proportion changes over time - the most extreme ponds retained their differences over a six years' study period. The predominance of different genotypes in different ponds could be a consequence of differential selection varying between ponds (selection hypothesis), or, alternatively, of different gamete production patterns among ponds (gamete pattern hypothesis). The selection hypothesis was tested in adults by a mark-recapture study in the 12 ponds. As the relative survival and proportion of LLR, LR and LRR did not correlate within ponds, this study provided no evidence for the selection hypothesis in adults. Then, both hypotheses were tested simultaneously in juvenile stages (eggs, tadpoles,

metamorphs and one-year old froglets) in three of the ponds. A gradual approach to adult genotype proportions through successive stages would support the selection hypotheses, whereas the presence of adult genotype proportions already at the egg stage would support the gamete pattern hypothesis. The result was a weak preference for the gamete pattern hypothesis. These results thus suggest that selection is of little importance for shaping genotype distributions of all-hybrid populations of *P. esculentus*, but that further studies are needed for a confirmation. Moreover, the study provided valuable data on genotype-specific body lengths, adult survival and sex ratios.

Chapter 4 analyzes the genetic structure and diversity of *P. esculentus* in the Danish archipelago and adjacent countries. This study is the first to cover the entire geographic range of Danish, Swedish and German all-hybrid populations, documenting their extension and providing a broad picture of their diversity of neutral genetic markers and genomotype proportions. With 18 microsatellite markers, it is demonstrated that genetic diversity declines northwards in agreement with the glacial refuge and central-marginal hypotheses; however populations on small and medium-sized islands are no less diverse than those on large islands and continental peninsulas. Isolation by distance exists across the archipelago, with limited influence of the fragmentation by brackish seawater. The extremely low genetic diversity in all-hybrid populations, compared to adjacent populations, might be responsible for the maintenance of their special breeding system. The study also demonstrates large variation in the proportions of LLR, LR and LRR genomotypes between ponds, but little geographic pattern in their distribution. Instead, relationships between the proportions of these genomotypes and some of the 15 ecological pond parameters monitored were found. Size differences between LLR, LR and LRR further suggest ecological differences.

Zusammenfassung

Hybridisation ist für die Evolution von grosser Wichtigkeit und hat verschiedene Konsequenzen. Eine davon ist die Bildung neuer taxonomischer Einheiten. Die meisten hybriden Pflanzen-Taxa sind sexuelle Arten, die durch Tetraploidisierung entstanden sind, während viele hybride Tierarten klonale Fortpflanzung haben und deshalb als evolutionäre Sackgassen betrachtet werden. Der Europäische Teichfrosch, *Pelophylax esculentus*, ist in seiner Biologie ein einmaliger Hybride, weil er in drei unterschiedlichen Paarungs-Systemen vorkommt. In zwei von ihnen pflanzt er sich klonal mit der einen oder anderen Eltern-Art fort. Im Unterschied dazu besteht das dritte Paarungs-System aus reinen Hybridpopulationen die 1) in der Fortpflanzung unabhängig von den Elternarten sind und 2) möglicherweise sexuelle Reproduktion mit genetischer Rekombination haben. Diese zwei Eigenschaften repräsentieren wichtige Schritte auf dem Weg zu Artbildung.

P. esculentus ist ein Hybrid zwischen dem Kleinen Teichfrosch, *P. lessonae* (Genotyp LL), und dem Seefrosch, *P. ridibundus* (Genotyp RR). In den reinen Hybrid-Populationen sind die Hybriden entweder diploid (LR) oder triploid (LLR oder LRR). LR-Frösche produzieren LR und/oder R-Gameten, LLR-Frösche produzieren L-Gameten, und LRR-Frösche produzieren R-Gameten. Für eine erfolgreiche Fortpflanzung müssen sich deshalb Frösche verschiedener Genotypen miteinander paaren. Die vorliegende Doktorarbeit besteht aus vier eigenständigen Kapiteln über reine Hybrid-Populationen. Alle zielen darauf ab, unser Verständnis über die Struktur und Dynamik reiner Hybrid-Populationen von *P. esculentus* zu erweitern.

In Kapitel 1 wird demonstriert, dass reine Hybrid-Populationen von diploiden (LR) und triploiden (LLR und LRR) *P. esculentus* sich tatsächlich sexuell fortpflanzen. Dies ist von evolutionärer Wichtigkeit für die Hybriden, denn klonale Fortpflanzung geht auf Kosten der genetischen Vielfalt und der Fähigkeit, schädliche Mutationen zu eliminieren. Mikrosatellit-Analysen von Eltern und Nachkommen aus einem Kreuzungs-Experiment zeigten, dass triploide Hybride beider Geschlechter und Genotypen (LLR und LRR) ihre homospezifischen Genome rekombinierten. In den untersuchten natürlichen Populationen hatte die grosse Mehrheit ein niedriges Multilocus-Kopplungsungleichgewicht (multilocus linkage disequilibrium), was auf eine hohe Rekombinationsrate hinweist. Wie aus Modellen zum Paarungssystem vorhergesagt, war das linkage disequilibrium für das L-Genom gleichbleibend tief, während das des R-Genoms mit zunehmendem Anteil rekombinierender

Triploiden abnahm. Diese direkte Evidenz für sexuelle Reproduktion bei *P. esculentus* erfordert eine Abkehr von der konventionellen Auffassung, hybridogenetische Taxa seien diploide Organismen mit klonaler Fortpflanzung. Sie können sexuell unabhängige Einheiten mit evolutionärem Potenzial sein.

Kapitel 2 liefert Daten und Interpretationen über Gameten-Typen und Geschlechtsbestimmung. Sie sind essentiell, um zu verstehen, wie dieses ungewöhnliche System funktioniert, welches evolutionäre Potenzial in ihm steckt und welchen Gefahren es ausgesetzt ist. Gameten-Muster bei Hybriden sind generell interessant, weil triploide Individuen oftmals eine Schlüsselrolle spielen in der Artenbildung durch Hybridisierung. Die Kenntnis der Gameten-Typen (d.h. von Ploidie und genetischer Zusammensetzung) sowie ein Verständnis der Stabilität bzw. Dynamik von Hybrid-Populationen mit triploiden Invididuen ist deshalb wichtig, um die Rolle der Hybridisierung in der Evolution zu erforschen. Das Sezieren von Metamorphen aus einem Kreuzungs-Experiment bestätigte, dass in reinen Hybridpopulationen die Geschlechtsbestimmung auf einem XX-XY-System beruht, wobei das Y an das L-Genom gebunden ist. Aus Mikrosatelliten-Analyse der gekreuzten Eltern und ihrer Nachkommen konnte auf die Häufigkeit der verschiedenen Gameten-Typen geschlossen werden: Triploide beider Geschlechter erzeugten hauptsächlich haploide Keimzellen mit demjenigen Genom, welches sie doppelt hatten; ausserdem produzierten LLR-Weibchen mittels Automixis ungefähr 10% LL-Gameten. LR-Frösche zeigten viel Variation in ihrer Gameten-Produktion: z.B. produzieren diploide Männchen in den meisten Populationen haploide R-Spermien; aber in LRR-reichen Populationen war die LR-Sperma-Produktion genügend hoch (22%), um den beobachteten – aber bisher nicht verstandenen - Anteil an LRR-Männchen erklären zu können. Diese Daten weisen darauf hin, dass in reinen Hybrid-Populationen unterschiedliche Fortpflanzungs-Systeme vorkommen. Auf Basis der gefundenen Gameten-Anteile wurde ein Modell konstruiert, das für verschiedene Populationstypen die Gleichgewichts-Verhältnisse zwischen den Genotypen berechnet. Die errechneten Gleichgewichte stimmten mit empirischen Befunden in der Literatur gut überein. Das Modell sagte ausserdem voraus, dass Tetraploidisierung vorkommen kann, wenn Überleben und Fruchtbarkeit von Männchen und Weibchens erhöht wird. Ob reine Hybridpopulationen durch die Einschleppung von Hybriden oder Individuen der Eltern-Arten gefährdet würden, weil das neue Genmaterial das Überleben von Nicht-Hybriden fördern würde, kann ohne weitere Kenntnisse über die Gründe der Sterblichkeit von Nicht-Hybriden nicht gesagt werden. Aber zumindest für R-Genome mit einem Y-Faktor kann vorhergesagt

werden, dass sie – falls eingeführt – invasiv sein und die genetische Last der reinen Hybridpopulationen erhöhen werden.

Kapitel 3 zielt darauf ab, die Wichtigkeit der Selektion für die Strukturierung reiner Hybrid-Populationen zu ermitteln. Der Einfluss differentieller, d.h. örtlich verschiedener, Selektion auf die geografische Verteilung von Genotypen in Hybrid-Systemen wird seit langem diskutiert, ist aber immer noch umstritten. Mit Daten von 12 schwedischen Teichen wird zunächst gezeigt dass – trotz signifikanten Änderungen in der Populations-Zusammensetzung über die Zeit – die Unterschiede zwischen Teichen mit den extremsten Genotyp-Verhältnissen erhalten bleiben. Das Vorherrschen verschiedener Genotypen in verschiedenen Teichen könnte zum einen eine Folge differentieller Selektion sein (Selektions-Hypothese); zum anderen könnte es auf unterschiedlichen Gameten-Produktions-Mustern beruhen (Gameten-Muster-Hypothese). Die Selektions-Hypothese wurde in den 12 Teichen an erwachsenen Fröschen mittels Fang-Markierung-Wiederfang-Methode getestet. Da die relativen Überlebensraten und Häufigkeiten der LLR, LR und LRR innerhalb von den Teichen nicht korrelierten, erbrachte diese Studie keinen Beleg für die Selektions-Hypothese. Sodann wurden in dreien der Teiche beide Hypothesen gleichzeitig an vier Juvenil-Stadien getestet (Eier, Kaulquappen, frisch metamorphosierte und einjährige Frösche). Eine allmähliche Veränderung der Genotyp-Verhältnisse von denen der Juvenilstadien zu denen der Adulten würde die Selektions-Hypothese stützen. Hingegen würde die Gameten-Muster-Hypothese gestützt, wenn die Genotyp-Verhältnisse bei den Adulten schon im Eistadium vorhanden wären. Das Resultat ergab eine schwache Bevorzugung für die Gameten-Muster-Hypothese. Dieser Befund legt nahe, dass unterschiedliche Selektion zwischen Teichen wenig Einfluss auf die Genotypen-Verhältnisses in reinen Hybrid-Populationen von *P. esculentus* hat, dass aber weitergehende Studien notwendig sind, um dies zu bestätigen. Darüber hinaus lieferte die Studie wertvolle Daten über Genotyp-spezifische Körperlänge, Überlebensraten bei Erwachsenen und Geschlechterverhältnis.

Kapitel 4 analysiert die genetische Struktur und Diversität von *P. esculentus* Populationen im dänischen Archipel und in benachbarten Ländern. Diese Studie ist die erste, die den gesamten geografischen Raum von dänischen, schwedischen und deutschen reinen Hybrid-Populationen abdeckt, ihre Ausdehnung und Genotyp-Verhältnisse dokumentiert und auf der Basis von neutralen genetischen Markern ein umfassendes Bild ihrer Diversität liefert. Mit 18 Mikrosatelliten-Markern wurde gezeigt, dass die genetische Vielfalt gegen Norden hin abnimmt – in Übereinstimmung mit der Eiszeitrefugien-Hypothese und der Idee eines Zentrum-Rand-Gradienten. Populationen auf kleinen und mittelgrossen Inseln sind jedoch

nicht weniger genetisch divers als diejenigen auf grossen Inseln und kontinentalen Halbinseln. Im gesamten Archipel besteht genetische Isolation durch Entfernung (isolation by distance), während die Fragmentierung der Populationen durch das schwach salzhaltige Wasser der Ostsee nur geringen Einfluss hat. Die äusserst geringe genetische Vielfalt in reinen Hybrid-Populationen, verglichen mit benachbarten Populationen, könnte für die Aufrechterhaltung ihres speziellen Fortpflanzungs-Systems verantwortlich sein. Ausserdem zeigt die Studie eine grosse Vielfalt in den Anteilen von LLR-, LR- und LRR-Genotypen zwischen den einzelnen Teichen, aber kein geografisches Muster in der Verbreitung von Populationen mit mehr oder weniger LR-, LLR- oder LRR-Tieren. Stattdessen wurden Zusammenhänge zwischen den Anteilen dieser Genotypen und einigen der fünfzehn gemessenen ökologischen Teich-Parametern gefunden. Auch die Grössenunterschiede zwischen LLR-, LR- und LRR-Tieren stehen in Einklang mit ökologischen Unterschieden.

General introduction

Hybridization

Hybridization has a variety of evolutionary consequences (Arnold 1992; Coyne & Orr 2004; Chapman & Burke 2007). First, if hybrids are unfit, reinforcement of reproductive isolation between the hybridizing species may take place and improve their ability to recognise and avoid each other as mates. Secondly, genes may pass from one parental species to the other by introgression. Thirdly, if the hybrids are fit in some environment and become reproductively isolated from the parental species, they can form a new species of hybrid origin.

The importance of hybridization as a source of new species has long been subject to heavy discussion (Bullini 1994; Arnold 1997; Mallet 2005). The discussion has focused on the frequency of hybridization and the fitness of the resulting hybrids. Earlier views were that hybridization was rare and that hybrids were less fit than the parental species and thus of no evolutionary importance (reviewed in Burke & Arnold 2001). More resent views state that in spite of a rarity on a per individual basis, hybridization is usually frequent on a per species basis, depending on the species definition (Mallet 2005). Per species occurrences of 1% (Schwenk et al. 2008) to 10% (Mallet 2005) have been estimated for animals and of 25% for plants (Mallet 2005). Furthermore, although most hybrids are unfit, the variation in their fitness is large, so that a small fraction of them may actually be fit (Arnold & Hodges 1995; Barton 2001; Burke & Arnold 2001) and have ecological and evolutionary importance.

New hybrid species can arise by either homoploid or polyploid speciation (Coyne & Orr 2004; Mallet 2007). In the most common form of homoploid (diploid) speciation, a chromosomal rearrangement happens, so that the hybrids cannot produce viable/fertile offspring with the parental species, but only with each other. In polyploid (tetraploid) speciation, the number of chromosomes is doubled so that each can pair with its copy. Backcrossings of tetraploid hybrids with the diploid parental species would yield triploid offspring, which are usually sterile, or inviable. Much evidence for a large contribution of hybridization to formation of new species now exists; especially for plants but also for animals (Arnold 1997; Hegarty and Hiscock 2005; Wissemann 2007).

While the above mechanisms lead to establishment of new, normal, sexual species with hybrid origin, some hybrids form clonal taxa. Clonal taxa are often the result of hybridization between species that are so divergent, that the chromosomes cannot pair properly during meiosis. Three kinds of reproductive modes exist in asexual hybrid taxa:

parthenogenesis, gynogenesis and hybridogenesis (Dawley 1989). Parthenogens are all-female taxa that lay unreduced eggs which hatch into new females that are genetically identical to their mothers. Gynogens are similar to parthenogens, but need sperm to activate – not fertilize – the eggs. Gynogens therefore have to mate with males of the parental or related species. Hybridogens discard the chromosome set from one of their parental species in their germ line and produce clonal, reduced gametes with the other chromosome set. For producing a new generation of hybrids, hybridogens thus have to mate with the parental species whose genome they discarded. Some hybridogens are all-female, whereas others comprise both sexes. Among vertebrates, parthenogenesis is found in reptiles, whereas the sperm-dependent modes, gynogenesis and hybridogenesis, are found in fishes and amphibians. As the centrosome is provided with the sperm in fish and amphibians, they cannot be parthenogenetic (Elinson 1989). Clonal reproduction is not known from birds and mammals, but relative common in invertebrates. Whereas clonal reproduction in vertebrates is thought to always be a result of hybridization (Vrijenhoek 1989; Kearney 2005), most clonal hybrid invertebrates are thought to have inherited clonal elements of reproduction from their parental species (Bullini & Nascetti 1990).

The concept of hybridogenesis has recently been expanded in connection with polyploid hybrids. Kleptonic hybridogenesis has been described in *Ambystoma* salamanders as a mostly gynogenetic reproduction mode, where sperm elements are occasionally incorporated into the offspring (Bogart et al. 2007). Besides, meiotic hybridogenesis has been described in the fish *Rutilus alburnoides* as a mode of hybridogenesis with triploid individuals providing genetic recombination (Alves et al. 1998). In both cases, polyploidy introduces elements of genetic recombination into this otherwise clonal inheritance of hybridogenetic animals. This is of crucial importance for the evolutionary potential of these taxa.

The diversity of reproductive modes makes hybrids exciting for biologists wanting to understand processes in evolution and speciation. About this topic, Vrijenhoek (1989) wrote: “Examination of unusual processes opens a window to understanding what is common and what is normal”. The European, edible frog, *P. esculentus*, is amazing because it forms a variety of different breeding systems and even has two hybridogenetic sister species. The edible frog is therefore not only well known as a delicacy in the French cuisine, but also as an attractive study object for evolutionary biologists.

P. esculentus systems

The edible frog, *P. esculentus*, (called *Rana esculenta* until Frost et al. 2006) is a hybrid between the pool frog, *P. lessonae* (genotype LL), and the marsh frog, *P. ridibundus* (genotype RR). Most hybrids, whether from primary hybridizations or from old hybrid lineages, are diploid LR that reproduce by normal diploid hybridogenesis. They produce clonal R gametes and are dependent on L gametes from *P. lessonae* for producing new hybrids (Graf & Polls Pelaz 1989). In this, so-called L-E system (*lessonae-esculentus* system), the R genome is thus the only permanent part of the hybrids and is clonally propagated. When hybrids mate among themselves, RR offspring arise, but die because they are homozygous for recessive deleterious mutations present on the clonal R genome (Vorburger 2001; Guex et al. 2002).

The breeding system investigated in the present study is the all-hybrid populations of *P. esculentus*. These populations are of particular interest, because they have acquired reproductive independence from the parental species and thus taken an important step towards speciation. All-hybrid populations contain not only diploid LR hybrids, but also triploid LLR and LRR (Figure 1). Normally, LLR of both sexes make L gametes, LRR of both sexes make R gametes, LR males make R sperm and LR females make LR and/or R eggs, however variations occur (Graf & Polls Pelaz 1989; Jakob 2007 chap. 5). Random mating among the genotypes gives rise to the original LLR, LR and LRR genotypes, but also to LL and RR, i.e. parental species genotypes. However, LL and RR normally die at an early stage and are never or only very rarely found as adults (Christiansen et al. 2005; Arioli 2007, chap. 3; Jakob 2007, chap 2). As the proportions of LLR, LR and LRR vary among ponds, it is, however, not clear whether all-hybrid populations belong to one or several genetically distinct population types.

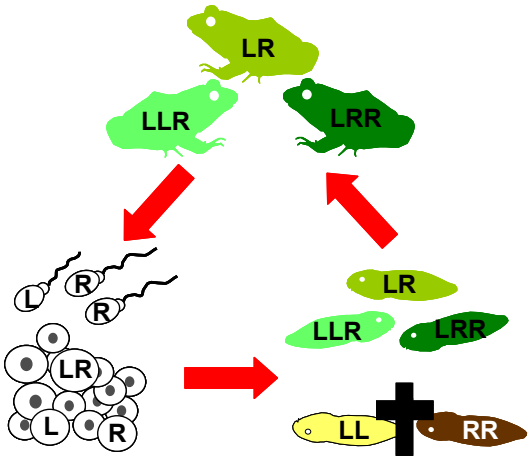

Figure 1. Reproduction in all-hybrid populations. See text.

The present PhD project

Although much work has been done on Scandinavian all-hybrid populations in recent years (Christiansen 2005; Christiansen et al. 2005; Arioli 2007; Jakob 2007), many questions about these interesting hybrids remained when the present PhD project started in 2005.

My primary desire was to find out, whether LLR frogs have genetic recombination between their two L chromosome sets, and whether LRR frogs recombine their two R chromosome sets. As the original *P. esculentus* breeding system was probably the diploid, clonal L-E system, where *P. esculentus* lives as a sexual parasite, the demonstration of genetic recombination in all-hybrid populations would indicate secondary attainment of sex after an intermediate asexual stage. This would be very exceptional, and would mean that *P. esculentus* in these all-hybrid populations had taken yet another step towards becoming a new species, rather than representing a clonal, evolutionary dead end.

The second task was to obtain a more detailed picture of the gametes produced, and confirm or reject the sex determination system expected for all-hybrid populations. This information was essential for understanding the dynamics and properties of the system, as both gamete content and sex determination should impact the genotype proportions in all-hybrid populations. Moreover, gamete patterns might be the key to explaining observed genotype proportion differences between ponds. By means of a model, equilibrium genotype proportions could be calculated, and analyses of the evolutionary potential of all-hybrid populations and the potential thereat by introduction of parental species could be made.

My third project was to investigate the temporal stability of all-hybrid populations in Sweden. Temporal stability, or at least consistency in differences between ponds, is a prerequisite for meaningful discussions of different population types or genotype proportions. At the same time, two new approaches were made to identify mechanisms producing different genotype proportions in different ponds.

Finally, I compared samples from all over the all-hybrid area in Denmark, Southern Sweden and Northern Germany in order to document and determine the extension of the all-hybrid populations in this area and investigate regional and ecological differences. As the area is an archipelago adjacent to the mainland, the impact of island size and isolation on the genetic diversity could be analysed. Furthermore, it was investigated weather all-hybrid populations had lower genetic diversity than the adjacent breeding systems in Germany and the Baltic states, to see if this could explain the death of parental species genotypes in all-hybrid populations. Lastly, correlations were sought between genotype proportions,

geographic locations and ecological parameters to elucidate why genotype proportions vary among ponds.

The majority of the data was collected by myself and field assistants during three field seasons in Scandinavia. Each year, I spent more than a month sampling adults in 12 Swedish ponds in spring and again in summer. In 2005, I additionally made a two months' sampling trip covering Danish all-hybrid populations as broadly as possible. In 2006 I performed a large crossing experiment in Sweden and collected eggs, tadpoles and also metamorphs in three natural ponds. All were raised to metamorphosis, i.e. for up to four months. The field season 2006 was shorter; the adult-sampling in the 12 ponds was only supplemented with sampling of one year-olds in three Swedish ponds. Additional data came from my own sampling trip to the Baltic states in 2004, several Germany sampling trips by Heinz-Ulrich Reyer and three previous years' sampling of the 12 Swedish ponds by Christian Jakob and Martina Arioli. After constructing a new microsatellite multiplex PCR procedure myself, our technician, Sandra Röthlisberger, took over the laboratory work whereas I scored all the alleles and determined the ploidy of the frogs based on dosage effect. The only exception is that Christian Jakob and Martina Arioli provided genotype data (LLR, LR and LRR) for some German, Baltic and their own Swedish samples.

The outcome of this PhD study is presented as four independent chapters following this general introduction. Two of the chapters have already been published as articles in international scientific journals, whereas two are not published yet. The many people that provided invaluable help at various stages are thanked in the acknowledgements of the individual articles. Here, I shall therefore only thank my husband, Daniel Leutwyler, and my parents, Hans and Lone Christiansen for their constant moral support, and my main supervisor, Heinz-Ulrich Reyer, for fruitful cooperation on many levels.

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Evolution 2009, vol 63, pages 1754-1768

From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs

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Abstract

Speciation via interspecific hybrids is very rare in animals, as compared to plants. Whereas most plants overcome the problem of meiosis between different chromosome sets by tetraploidization, animal hybrids often escape hybrid sterility by clonal reproduction. This comes at the expense of genetic diversity and the ability to purge deleterious mutations. However, here we show that all-hybrid populations of diploid (LR) and triploid (LLR and LRR) water frogs (*P. esculentus*) have secondarily acquired sexual reproduction. First, in a crossing experiment analyzed with microsatellite markers, triploid hybrids of both sexes and genotypes (LLR and LRR) recombined their homospecific genomes. Second, the great majority of natural populations investigated had low multilocus linkage disequilibrium, indicating a high recombination rate. As predicted from mating system models, the L genome had constant, low levels of linkage disequilibrium, while linkage disequilibrium in the R genome showed a significant reduction with increasing proportion of recombining triploids. This direct evidence of sexual reproduction in *P. esculentus* calls for a change of the conventional view of hybridogens as clonally reproducing diploids. Rather, hybridogens can be independent sexually reproducing units with an evolutionary potential.

Introduction

Hybridization instantly creates individuals with a new genetic composition and is therefore a potentially powerful force in evolution (Jiggins et al. 2008; Wissemann 2007). Whether hybridization leads to speciation depends on the hybrids' ability to survive and reproduce (Arnold and Hodges 1995; Barton 2001; Chapman and Burke 2007). Two reproductive challenges need to be overcome for establishment of new hybrid taxa: First, the hybrids must

be fertile, in spite of having two dissimilar chromosome sets which might interrupt meiosis (Arnold and Hodges 1995; Chapman and Burke 2007). Second, the hybrids must be spatially or reproductively isolated from the parental species (Chapman and Burke 2007; James and Abbott 2005; Wang et al. 2001).

Normal meiosis, as well as reproductive isolation, can instantly be restored by tetraploidization. Via this process, hybridization has had a large impact on plant evolution (Arnold 1997; Hegarty and Hiscock 2005; Wissemann 2007), while in animals, remarkably few examples of tetraploid speciation are known (Orr 1990; Otto and Whitton 2000). In animal hybrids, fertility and reproductive isolation are, however, often established by different kinds of clonal reproduction which may or may not be accompanied by polyploidy. Among clonal vertebrates, reptiles are parthenogenetic, while fishes and amphibians depend on sperm from a sexual species for initiating embryogenesis (Vrijenhoek et al. 1989): In gynogenetic taxa, the sperm activates, but usually does not fertilize the eggs. In hybridogenetic taxa, fertilization takes place; yet, there is normally no recombination between the parental genomes. This is because the paternal genome is usually excluded from the germ line prior to meiosis while the remaining maternal genome is transmitted clonally (Dawley 1989). Hybridity is restored each generation by matings with the paternal species. The hybrids' soma is thus made up by both the sexual paternal and the clonal maternal genome, while the hybrids' germ line contain only the latter.

For hybrid speciation to be of evolutionary importance, a third factor is crucial: genetic recombination. Genetic recombination via sexual reproduction enhances genetic diversity and is generally agreed to convey three important benefits: One, high genetic diversity is required for defence against fast evolving parasites (Red Queen hypotehsis, Hamilton 1980). Two, the combination of beneficial mutations from different individuals enhances the efficiency of selection (e.g. Colegrave 2002; Cooper 2007; Fisher 1930). Three, and most importantly, the combination of deleterious mutations allows their purging from the population (Muller 1932; e.g. Vrijenhoek 1994). Without recombination, clonal lines are predicted to accumulate deleterious mutations via Muller's ratchet, which will eventually lead to their extinction.

As a consequence of their clonal reproduction modes, parthenogenetic, gynogenetic and hybridogenetic hybrid animal taxa lack the above mentioned advantages of genetic diversity and the ability to purge mutations. Hence, they are generally considered to be "evolutionary dead ends", at least as far as individual lineages are concerned (e.g. Maynard Smith 1992; e.g. Vrijenhoek et al. 1989). In agreement with this, strictly clonal taxa are, with

very few exceptions (Butlin 2002), distributed as short-lived tips on the tree of life mainly comprised of sexual taxa (Simon et al. 2003).

However, at least genetic diversity seems to be higher in clonally reproducing taxa than previously assumed, and various mechanisms have been described how this can be achieved. First, clonal hybrids often arise recurrently from different progenitors. Hence, they have a high genetic diversity possibly enabling them to fit different ecological niches (frozen niche variation hypothesis, Vrijenhoek 1984). Recurrent origin of clonal and polyploid sexual lineages is known from several plants (Soltis and Soltis 1999) and also from animals, including ostracods (Little and Hebert 1997), fishes (Janko et al. 2003; Pala and Coelho 2005), reptiles (Moritz et al. 1989) and some anurans (Ptacek et al. 1994; Stöck et al. 2005). Second, some allegedly asexual organisms are not strictly clonal but occasionally incorporate new nuclear material from a sexual host (Hedges et al. 1992; Schartl et al. 1995; Spolsky et al. 1992). The most recent discovery of such a mechanism is "kleptogenesis" in unisexual salamanders of the genus *Ambystoma* (Bogart et al. 2007): all-female lines can incorporate (parts of) nuclear genomes from sperm from sympatric sexual species and presumably later discard other parts of the genome. Third, in bisexual hybridogenetic species, like the edible frog, *Pelophylax esculentus* (called *Rana esculenta* until Frost et al. 2006), and the Iberian minnow, *Squalius alburnoides*, hybrid x hybrid matings lead to offspring with parental genotype (cf. Fig. 1b). Although rarely viable, these offspring could recombine the otherwise clonal genomes if they succeed in reproducing (Alves et al. 1998; Hotz et al. 1992; Vorburger 2001c). While the existence of these three mechanisms can not be denied, their potential for lifting the doom of "evolutionary dead end" from the relevant hybrid taxa is subject to discussion.

Here we investigate the potential for systematic and frequent sexual reproduction in hybridogens through a mechanism called meiotic hybridogenesis. The term refers to the possibility that in polyploid hybridogens of the general type AAB, the homospecific chromosome sets from one parental species, A, recombine in a normal meiosis, whereas the set from the other parental species, B, is discarded (Alves et al. 1998). Preferential pairing of homologous chromosomes and elimination of the unmatched chromosomes has been shown for a number of triploid fish and frog hybrids (see Morishima et al. 2008 and references therein) but, so far, clear evidence for recombination through meiotic hybridogenesis comes from one species only: the Iberian minnow, *S. alburnoides* (Crespo-Lopez et al. 2006).

It might be argued that meiotic hybridogenesis is a rare and special phenomenon without much general relevance for the role of hybrids in animal evolution. However, meiotic

hybridogenesis is interesting as a newly discovered possibility for hybridogenetic hybrids to obtain recombination in a regular, non-accidental way. Besides, the list of taxa with meiotic hybridogenesis will surely grow: Firstly, with the increasing application of molecular tools to organisms from different populations, the list of known hybridogens has grown recently and is likely to grow further. Secondly, since hybridogenesis was originally discovered in the diploid topminnow, *Poeciliopsis monacha-lucida* (Schultz 1969), polyploidy in hybridogens increasingly appears to be the rule, rather than the exception. At present, polyploidy is known from four of the six genera with hybridogenesis. Water frogs (*Pelophylax*, Berger 1967), Iberian minnows (*Squalius*, Carmona et al. 1997), spined loaches (*Cobitis*, Saitoh et al. 2004) and Oriental weatherloaches (*Misgurnus*, Morishima et al. 2008) excibit polyploidy while only hybridogenetic topminnows (*Poeciliopsis*) and stick insects (*Bacillus*, Bullini and Nascetti 1990) are purely diploid. Polyploidy is also known from a hybridogenesis-related mode of reproduction in the Batura toad (*Bufo viridis* complex, Stöck et al. 2002). We are thus just at the beginning of discovering the diversity and implications of hybrid reproduction modes.

Hence, investigating the extent of recombination during meiotic hybridogenesis and thus the long-term evolutionary potential for intraspecific hybrids seems timely and potentially relevant for more species than presently assumed. The edible frog, *Pelophylax esculentus*, provides a particularly interesting system for such an investigation, because it is the only hybrid yet known also to form self-sustaining, hybridogenetic, all-hybrid populations. In the absence of the parental species, meiotic hybridogenesis is the sole potential source of frequent recombination and could thus be of crucial evolutionary importance for these populations. Moreover, *P. esculentus* comes in various mating systems and, hence, offers an opportunity to study successive stages of incipient hybrid speciation.

The *Pelophylax esculentus* systems

Pelophylax esculentus (*Rana esculenta*) originated, and still originates, from interspecific matings between the two sexual water frog species, *P. lessonae* (the pool frog, genotype LL) and *P. ridibundus* (the marsh frog, genotype RR). The parental species, as well as the diploid *P. esculentus* hybrid with the genomic composition LR, have wide distributions in Europe. In the western part of this distribution area, LR excludes the L genome from the germ line prior to meiosis and transmits the R genome to the gametes clonally. As a result, matings between hybrids yield RR offspring, but these typically die due to homozygosity for deleterious mutations in the clonal R genome (Guex et al. 2002; Vorburger 2001a and references therein).

In order to form a new generation of hybrid LR, *P. esculentus* is dependent on L gametes obtained from mating with *P. lessonae* (LE system, Fig. 1). In parts of Eastern Europe the pattern is reversed: hybrid LR excludes the R genome, produces L gametes and, therefore, lives in sympatry and mates with *P. ridibundus* (RE system). In both of these diploid systems, (reviewed by Graf and Polls Pelaz 1989) *P. esculentus* face disadvantages with respect to both of its genomes: the one in the hybrid's germ line is clonal, while the other, sexual, genome must for every generation be obtained by mating with the parental species. Various LE, RE systems and *lessonae-esculentus-ridibundus* populations with both diploid and triploid *P. esculentus* also exist (Günther 1991; Rybacki and Berger 2001; Tunner and Heppich-Tunner 1992), but unfortunately hardly anything is known about how these diverse and complicated populations function.

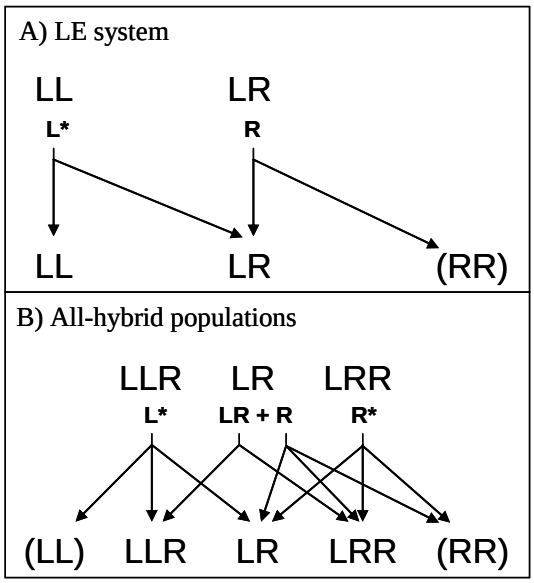

Figure 1. Adults, gametes and offspring of a) the LE system with *P. lessonae* and *P. esculentus* and b) all-hybrid populations of *P. esculentus*. * denotes gametes that could be recombined. Non-hybrid offspring from intraspecific *P. esculentus* matings are in parenthesis because they typically die before reproductive maturity. Note that in the LE system, the R genome is never recombined and the L genome is provided anew in every generation by *P. lessonae*. In the all-hybrid populations, both L and R genomes are supplied by hybrids and would regularly undergo recombination, if triploids have meiotic hybridogenesis.

The present study focuses on all-hybrid populations of *P. esculentus* (EE system) that, by definition, live and reproduce without any of the parental species. Thus, the propagation of both parental genomes, as well as any recombination within them, must be undertaken by hybrids alone. All-hybrid populations are found in large areas of Denmark, southern Sweden north-eastern Germany, and patchily in northern Poland and probably a few localities in south eastern Europe (Arioli 2007 chap. 5; Christiansen et al. 2005; Mikulícek and Kotlík 2001; Rybacki and Berger 2001); (reviewed by Plötner 2005). These populations consist of diploid (LR) and one or two types of triploid hybrids (LLR and LRR). LLR frogs of both sexes provide L gametes while LRR make R gametes. Within the diploid LR, all males and some females produce R gametes, while all females and a few males make unreduced LR gametes yielding new triploids upon fusion with haploid gametes (Arioli 2007 chap. 1; Christiansen et

al. 2005; Graf and Polls Pelaz 1989; Jakob 2007 chap. 5). Sex determination is an xx-xy system with a dominant male-determining y factor. The y factor is supposed to be present in the L genome only (Berger and Günther 1991-1992; Graf and Polls Pelaz 1989), which means that L genomes are either L_x or L_y while all R genomes are R_x. As a consequence, LLR and LR come in both sexes, while the great majority of LRR are females (Jakob 2007 chap. 2 and the present study). In this way, the mix of di- and triploid hybrid frogs form self-sustaining populations producing all gametes needed for a new generation of similar composition (Fig. 1). Non-hybrid LL and RR offspring are also formed, but die off in natural ponds during the tadpole stage (Arioli 2007 chap. 3).

In all three systems, clonally propagated *P. esculentus* genomes face the risk of mutation accumulation. In the LE and RE systems, some accumulation can be tolerated, as the clonal genome is constantly paired with a healthy parental genome in the hemiclonal hybrids (confirmed in LE by Vorburger 2001b). Nevertheless, the lifespan of the clonal genomes in diploid systems appears limited, as old clones are likely to become inviable or replaced by new genomes that were more recently derived from primary hybridization between the parental species. In the all-hybrid populations, the situation was, so far, unknown. It was often assumed that the LLR recombine their two L chromosome sets after exclusion of the R, and that, likewise, LRR recombine their two R sets after exclusion of the L genome (Graf and Polls Pelaz 1989; Günther et al. 1979; Som and Reyer 2006a). Under this assumption, the all-hybrid populations might be functionally sexual with a higher evolutionary potential than diploid LE and RE system populations. However, experimental evidence for recombination in triploids is scarce and controversial, due to low availability of polymorphic genetic markers. Based on allozyme and sex data, Günther et al. (1979) probably found recombination in one Polish LRR male (table 5, cross 25/26). Furthermore, Arioli (2007 chap. 1), using microsatellite analysis on Swedish frogs, detected recombination in an LRR female, but not in an LLR male. While these data demonstrate the capability of triploids to recombine, it remains unclear whether recombination happens as a rule or as an exception and whether there are sex- and/or genotype- (LLR vs. LRR) specific differences in the recombination rate.

Here we present the first crossing experiment with a sufficient number of frogs (30) and polymorphic genetic markers (18) to conclude that intragenomic recombination takes place in triploids of both sexes and genotypes (LLR and LRR). We also provide previously unpublished microsatellite primers and new multiplex PCR protocols.

Confirming recombination in triploids does, however, not suffice to conclude that all-hybrid populations are functionally sexual. Therefore, assessment of the impact of triploid-

mediated recombination on the genetic structure of the L and R genomes in wild populations was needed. One might expect populations with many triploids to be highly recombined and thus have low multilocus linkage disequilibrium. This, however, should be true only for the R genome; not for the L genome. The reason for the difference is that R gametes can originate from both recombining LRR and non-recombining LR frogs whereas L gametes come from recombining LLR frogs alone (see Fig. 1). The monopoly on L gamete production guarantees LLR frogs a large and constant reproductive contribution to the next generation and, hence, should result in high recombination rates of L genomes, irrespective of the LLR/(LLR+LR) ratio. This prediction was previously confirmed by a mathematical model (Som and Reyer 2006a), but empirical data are lacking. In contrast, recombination rates of R genomes should, on average, be lower but increase with LRR/(LRR+LR) ratios. For this prediction, neither theoretical nor empirical studies were available.

Here we show that linkage disequilibrium was low in a large sample of natural populations from across the Danish and Swedish range, indicating that natural recombination rates are sufficiently high for these all-hybrid populations to be functionally sexual. We also provide evidence for the expected correlations between linkage disequilibrium and population structure. Finally, we confirm that pond-specific influences and method-specific biases were without importance for these results. In conclusion, the all-hybrid populations are an example of a hybridogen that, in a unique way, has become an independent evolutionary unit with sexual reproduction and thus a long-term evolutionary potential.

Methods

Overview

The study was carried out on Swedish and Danish all-hybrid populations, because these are geographically isolated from populations with parental species (Christiansen et al. 2005; Jakob 2007 chap. 2).

For direct evidence of whether triploids recombine, adult frogs were sampled, genotyped and crossed and the offspring were reared and genotyped. Then, segregation and linkage analyses were performed on the inheritance pattern of the microsatellite alleles analyzed. Absence of linkage between the majority of loci, when compared pairwise, would indicate recombination.

For investigating the level of recombination in natural populations, frogs were sampled in ponds with different proportions of clone-propagating diploid (LR) and recombining triploid frogs (LLR and LRR). All individuals were genotyped, and the

multilocus linkage disequilibria in the L and R genomes were calculated as $\bar{r}_d$ for each pond separately. Low $\bar{r}_d$ values would indicate high levels of recombination. The effects of genome, population structure, pond-specific effects and method-specific biases on $\bar{r}_d$ were investigated to test the predictions outlined in the introduction and to test suspicions of artifacts. Finally, F statistics were calculated, because non-random mating, resulting in high F_{IS} values, would also affect $\bar{r}_d$.

Crosses

Genetic variation in Swedish and Danish *P. esculentus* is very low (Arioli 2007 chap. 4; Christiansen et al. 2005). To obtain genetic data at multiple heterozygous loci for linkage analysis, it was therefore necessary to: 1) Screen both published and unpublished microsatellites for polymorphism in Scandinavia and design multiplex PCRs with the final selection of 18 primer pairs. 2) Select the most heterozygous triploids of a large sample of frogs for crossing. 3) Raise the larvae to an age where offspring genotypes could be inferred reliably when the heterozygous parents shared an allele. When parents share one allele, alleles or even whole chromosomes missing in the offspring can lead to misinterpretation of the parental contributions. This was of real concern, because many young larvae are aneuploid, i.e. they have mixed, uninterpretable genotypes with extra or missing alleles (Christiansen et al. 2005 and unpublished data from the present study). Raising the larvae to metamorphosis ensured that most aneuploid offspring died off and did not enter the analyses.

Crossing and rearing took place at Stensoffa Field Station, Scania, Sweden. Between May 12 and 22, 2006, i.e. after their emergence from hibernation and before breeding, 269 frogs were caught at night using flashlight and dip net in one of the Danish (Alsønderup in Christiansen et al. 2005) and 10 of the Swedish ponds included in the investigation of natural populations described below. The frogs were marked individually with a transponder (Trovan ID101, Euro I.D., DE), toe-clipped for DNA analysis, and kept at approximately 7° C while the DNA samples were sent to the University of Zürich and analyzed for genome composition (LLR, LR, LRR) and heterozygosity (at LL in LLR and RR in LRR). The triploids with most heterozygous loci were preferred for the crossings, because recombination can only be assessed from combinations of heterozygous loci. This preference made a balanced design of source ponds impossible. Since males were more common than females among LLR frogs and females were predominant among LRR frogs, LLR males and LRR females were picked from a larger sample and were therefore more heterozygous than LLR females and LRR

males. Most L genome data therefore derived from males and most R genome data from females.

Six crossing tables were designed, each having 3-4 females and 5-6 males including at least one LLR, LR and LRR female and at least one LLR, two LR and one LRR male. Substitute frogs were added if the sperm or egg quality looked suboptimal. All females were crossed with all males within the same crossing table, so that all frogs were crossed to all genotypes (half-sib design).

Offspring were produced on May 30, 2006 by artificial fertilization as described by Berger *et al.* (1994). Sperm solutions from the testes of hormone-injected males were distributed into 3-5 petri dishes per male. Eggs were then gently squeezed out of the hormone-treated females and dropped directly into the individual sperm solutions of the 5-6 different males, in small portions and in random order. The following day, the egg clumps were transferred to 1 liter tubs with 1-2 cm of water and subdivided for better oxygen supply.

The water was changed every 2-4 days and the egg jelly was removed after hatching. On June 12, when most tadpoles had just reached the feeding stage, 15 healthy-looking tadpoles (or fewer, if 15 were not available) from each sibship were randomly selected for rearing in 40 liters outdoors tubs covered with mesh lids allowing air and sunlight through, but keeping predators out. Algae growing on the insides of the tubs, supplemented with rodent pellets, ensured food *ad libitum*. Filamentous algae were regularly removed, fowling water exchanged and Daphnia *sp*. added for good water quality. The tadpoles metamorphosed from July 18 onwards. Slow-growing tadpoles were eventually moved indoors into smaller tubs, where the last ones metamorphosed in mid October. Offspring that died early during rearing disappeared, while offspring that died as metamorphs or nearly metamorphosing tadpoles were attempted DNA-analyzed although they were sometimes rotten. In total, 1628 tadpoles were selected for rearing, DNA samples were obtained from 1487 offspring (91%), and 1463 offspring (90%) were successfully genotyped.

Natural populations

Population structure was investigated in 54 Danish and 12 Swedish ponds from mid May to mid August 2005. The Danish ponds were chosen as pairs of ecologically distinct ponds, maximally 5 km apart, from across the area of distribution. At each location, approximately 30 frogs (predominately adults) were caught at night with flashlight and dip net, were measured and had a toe tip cut for DNA analysis before being returned to their pond.

The Swedish ponds constituted 11 ecologically variable ponds in the center of the small distribution area in Scania, Southern Sweden, and one from a satellite population near Malmö, 18 km west of the others ("core ponds" in Jakob 2007 chap. 2). The Swedish ponds were sampled as described above, but in both May and August, and the frogs were additionally marked with a transponder for individual identification. The Swedish samples are thus the sum of different individuals from the two catching rounds.

In total, 2296 Danish and Swedish frogs were caught and genotyped.

Laboratory protocols

DNA from the ethanol-stored toe-tips was extracted with Qiagen BioSprint 96 DNA Blood Kit following Qiagen's protocol for tissue extraction. All samples were subjected to two PCRs with nine primer pairs each. The reactions were of 5 µl and contained 0.8 µl DNA extraction, 2.5 µl Qiagen Multiplex PCR Master mix and 1.7 µl primer mix. PCR 1 contained primers Res16, Res20 (Zeisset et al. 2000), RlCA5, RlCA1b5 (Garner et al. 2000), Ca1b6, Ga1a19, Re2CAGA3 (Arioli 2007 chap. 4), RlCA2a34 and Rrid064A (Table 1). PCR 2 contained Res22 (Zeisset et al. 2000), RlCA18 (Garner et al. 2000), Rrid013A (Hotz et al. 2001), Rrid059A redesigned (Hotz et al. 2001 and table 1: forward primer redesigned to extend the fragment amplified by 177 base pairs), Re1CAGA10 (Arioli 2007 chap. 4), RlCA1a27, ReGA1a23, Rrid169A and Rrid135A (Table 1). Both forward and reverse primers appeared in 0.1 µM (or rarer 0.2 µM) in the PCR. Of the forward primers, 8-40% were color labeled with FAM, VIC, NED or PET. PCR 1 was given 15 min of initial denaturation at 95° C, 30 cycles of 30s at 94° C, 90s at 57° C and 60s at 72° C and a final extension of 30 min at

Table 1. Primer sequences not previously published.

Locus	Sequence 5' - 3'	Repeat	Genome specificity	Genbank ass. no.	Sequenced by
ReGA1a23	F: ATT GCT TTG GCA GTG AAG G R: TGA CAT CAC AGT GGG AGG AG	GA_n	L	EU445523	Garner *et al.* , Arioli & Jakob
RlCA1a27	F: CAA ATG GGT CAT CCA CAC C R: GTT CAA GGG GGT CGA AAT AC	CA_n	L	EU445522	Garner *et al.*
RlCA2a34	F: GCT CCA TGC CAA AAG TCT TC R: TTG GGT ATG ATA CTA CAA GCT ATG C	GT_n	L +R[1]	EU445521	Garner *et al.*
Rrid059A redesigned	F: TTG GAG ACA GAC TTC CGT AGG	CA_n	L[1]+R	FJ024048	Hotz *et al.*
Rrid064A	F: TGT ACG GGC CTT TAG ACT GG R: AAC TTT TTG AAG GCC CCT TG	GT_n… …TA_nGT_n	R	EU445524	Hotz *et al.*
Rrid135A	F: TCT TTT GTT TTA GCG CAC CT R: CTG CCC GTC TAA GCA AGT GT	CA_nTA_n	R	EU445526	Hotz *et al.*
Rrid169A	F: CGG AAC TCC GCT TTA ATC AC R: CCC ATG TTG TCG TTG AGC TA	TA_n…CA_n	R	EU445525	Hotz *et al.*

[1] monomorphic in this genome

60° C. PCR 2 was run similarly, but with 31 cycles with 60° C in stead of 57° C. 0.7µl of the PCR products were run on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ) and the alleles were scored with the Genemapper software (Applied Biosystems 2004).

Genotyping

All samples were analyzed with 18 primer pairs amplifying loci in either the L genome, the R genome or both. The 18 primers were scored at a total of 13 loci in each genome. With some primers, genome specificity changed slightly with PCR conditions, i.e. typically monomorphic L-specific alleles could arise or disappear beside the R allele(s) according to annealing temperature or primer concentrations. However, monomorphic loci conveyed no information of importance for the present study, and the choice of scoring or leaving out particular loci for technical reasons would not bias the data on homozygosity/heterozygosity which was the focus of this study.

All alleles scored were specific to either the L or the R genome. Allele specificity was confirmed in *P. lessonae*, *P. esculentus* and *P. ridibundus* from Estonia, Latvia and Lithuania (unpublished data), in non-hybrid LL and RR offspring from the crossings and through the distribution of L and R specific alleles on LLR, LR and LRR frogs. Preliminary data from German and Swiss samples indicated, however, that in these more southern populations with higher genetic polymorphism, certain alleles were not genome-specific.

Four of the primer pairs (Res16, RlCA1b5, Ca1b6 and Ga1a19) amplifying both L and R specific alleles were used to distinguish LLR, LR and LRR frogs by dosage effect, i.e. by the relative intensities (peak heights) of the L and R alleles amplified (see Christiansen 2005). L:R peak heights were evaluated separately per 96-well PCR, both per locus and per allele combination within that locus. The great majority of the L:R peak height ratios clustered into discrete groups corresponding to the LLR, LR and LRR genotypes. Samples producing intermediate or extreme L:R ratios were subjected to repeated PCR analyses until each of the four dosage effect loci clearly signaled LLR, LR or LRR. Assignment to LLR, LR or LRR was thus determined independently at four loci. In non-hybrid offspring (LLL, LL, RR, RRR) the peak height ratios of heterozygous L or R loci were used to determine ploidy in the same way as just described. Not all loci and allele combinations proved diagnostic, but most did.

Samples that repeatedly gave conflicting results on genotype, i.e. had extra or missing alleles at particular loci, were classified as mixed genotypes. Mixed genotypes, constituted 3.6% of the crossing experiment offspring and 2.1% (2.7% inclusive null alleles, see below)

of the natural pond samples and were excluded from data sets where the relevant loci could not be scored unambiguously.

Null alleles, i.e. alleles missing according to the overall ploidy of the individual, can be a nuisance in population genetics, because in high frequencies they bias estimates of allele frequencies and heterozygosity. However, in this study, they were generally not a problem, as they were often directly detectable and occurred in low frequencies only. The adults used for crossings carried no problematic null alleles, as the analyses were made on the loci where they were heterozygous for real alleles. Spontaneously missing alleles in mutant crossing experiment offspring, as well as null alleles in the frogs from the natural populations, were all directly detectable at the four dosage effect loci, and on average half of them were unmasked and detectable in a hemizygous state at the remaining loci. For example, a null allele at an L locus without dosage effect would be masked in LLR frogs but unmasked in LR and LRR frogs. Individuals with detected null alleles were handled as mixed genotypes described above. Only in two ponds was the same locus found missing in more than two frogs (i.e. six and eight frogs respectively), indicating that undetected null alleles could occur at potentially problematic frequencies in these ponds. In one of the two ponds, the entire locus was therefore recoded as missing data. In the second pond, all individuals were hemizygous at that locus, so that the null allele could always be detected. It was therefore coded as a real allele.

For determining LLR and LRR proportions in the ponds, mixed genotypes were assigned to the most similar euploid genotype.

Statistics: crossings

The crossings yielded data from on 30 triploid frogs for segregation and linkage analyses. For males, the analyses were based on 19-58 (mean 41) offspring and for females on 30-86 (mean 66) offspring, as females were on average mated to more partners than males.

Non-random segregation would indicate selection during the experiment or unexpected genetic mechanisms. To check for random segregation at the heterozygous loci in the parents, offspring allele frequencies were tested with Chi-square tests for homogeneity with Yate's correction for continuity (Fowler and Cohen 1992). To correct for multiple tests (n = 55 L and 57 R loci), sequential Bonferroni correction of the P values was calculated according to Holm (1979) in the program MacBonferroni (Watkins 2002).

Linkage analysis involves analysis of the inheritance pattern at two loci that are heterozygous in a parent (e.g. Aa+Bb). *Without* recombination, all pairs of loci should show complete linkage, i.e. only two of the parent's allele combinations should be observed in the

offspring (e.g. A+B and a+b). In contrast, *with* recombination all four possible combinations should be found in the offspring (A+B, a+b, A+b and a+B) in approximately equal proportions of 0.25. Intermediate results, where the recombinant allele combinations (A+b and a+B) are significantly less frequent than the parental ones (A+B and a+b), would indicate reduced recombination and would be hard to explain if deriving from the majority the locus pairs. However, a few locus pairs must, by chance, be expected to have reduced or no recombination, due to physical linkage. Linkage was investigated with 2x2 Chi-square tests with Yate's correction for continuity (Fowler and Cohen 1992) for every pairwise combination of loci that were heterozygous in the parent.

Statistics: natural populations

The rate of recombination is not easily measured directly. Instead, linkage disequilibrium between multiple genetic markers was used for an indirect measure, as recombination and linkage disequilibrium should be negatively related (see the discussion). Pairwise and multilocus linkage disequilibria in natural populations were calculated as $\bar{r}_d$, as recommended by Halkett *et al.* (2005). $\bar{r}_d$ is an index of association adjusted for unequal sample size, calculated by the program Multilocus (Agapow and Burt 2001). First, L and R loci were divided into separate datasets. Then, the two homospecific allele sets in triploids were split up into haploid data by recoding all but one randomly chosen heterozygous locus into missing data. Recoding heterozygous loci into missing data is also how Multilocus handles diploid data, according to the documentation file. Calculations were based on 20-71 (mean 37) haplotypes in Danish ponds and 56-110 (mean 78) in Swedish ponds. One pond was excluded from the L and another from the R data set because less than our predefined minimum of 20 haploid genotypes had been sampled. Two further ponds were excluded from the L data and eight from the R data because no or only one locus was polymorphic. After that, the genomes had 2-11 variable loci (mean 3.8 for the L and 5.2 for the R), i.e. loci with at least 5 undeleted copies of an alternative allele.

Pairwise $\bar{r}_d$ was calculated in order check for locus pairs producing $\bar{r}_d$ values differing significantly from the mean $\bar{r}_d$ of the remaining pairs, when tested pairwise (locus pair in question vs. mean of remaining locus pairs) over all ponds. This pairwise within-pond approach was necessary because overall linkage was expected to differ between ponds. Significantly elevated linkage disequilibria could suggest physical linkage between the loci in question, whereas linkage disequilibria lower than the mean would be difficult to explain.

Multilocus $\bar{r}_d$ were calculated for each genome in each pond to test the predicted correlations between recombination and population structure outlined in the introduction. All linear regressions, correlations and t-tests were performed in SPSS (2004). The L and R slopes from the linear regressions were subjected to a test for difference between two regression lines (Fowler and Cohen 1992).

The expected relationships between linkage disequilibrium and population structure could be obscured by strong between-pond variation in the forces responsible for linkage disequilibrium, i.e. founder effect, drift, migration and ecological selection on linked loci. If these forces affect the L and R genomes to a similar extent, the magnitude of this problem might be revealed by the degree of correlation between linkage disequilibrium in the L and R genomes in the ponds. To test for such pond-specific effects, we correlated $\bar{r}_d$ values for the L and R genome.

Genetic diversity varied between ponds and was generally lower in the L-specific than the R-specific markers. To investigate whether the estimates of multilocus linkage disequilibrium were affected by this variation in genetic diversity, we tested for correlation between $\bar{r}_d$ and genetic diversity measured as expected heterozygosity summed over all loci per genome. Expected heterozygosity was for each locus calculated as $H_E = 1 - (al_1^2 + al_2^2 + al_3^2\ldots)$ from allele frequencies (al_1, al_2, al_3 etc) computed by the software, SPAGeDi (see below).

As mentioned above, all but one of the heterozygous loci in triploid frogs had to be excluded for the constructing haplotypes forcalculating $\bar{r}_d$. This affected the R genome the most, as its higher genetic diversity resulted in many R-heterozygous LRR frogs. Ponds rich in LRR frogs could thus theoretically have lower $\bar{r}_d$ values as a result of the lower resolution after the exclusion of the many heterozygous loci. To investigate whether $\bar{r}_d$ was affected by the resolution, it was tested whether the $\bar{r}_d$ values for the R genome were correlation with the number of hemizygotes (LLR and LR which had no loci excluded) in the sample they were calculated from.

To investigate inbreeding and population structuring, F_{IS}, F_{ST} and F_{IT} were calculated in the program SPAGeDi (Hardy and Vekemans 2002), which accepts a mixture of different ploidy levels. These F statistics were calculated for each genome separately so that with respect to the L genome, LLR provided diploid data while LR and LRR provided haploid data. Similarly, LLR and LR gave haploid R data while LRR gave diploid R data. Excluding all haploid data from the analyses had very little effect on the results, though.

Results

Crosses

Recombination data was obtained from 7 LLR females, 10 LLR males, 7 LRR females and 6 LRR males. Due to multiple heterozygosity, most individuals provided data for several pairwise locus combinations. The LLR frogs provided recombination data for a total of 18 out of 21 possible pairwise combinations of 7 polymorphic L loci, and the LRR frogs for 47 of 66 possible combinations of 12 polymorphic R loci. All heterozygous loci in these triploids demonstrated random segregation, i.e. none of the allele proportions differed significantly from 0.5 at the 0.05 significance level after sequential Bonferroni correction performed within each genome separately. All triploids produced three or four gamete types per locus pair, corresponding to the two parental types and one or both recombinant types. All triploids thus recombined all their loci, and only for one locus pair were not all four gamete types present.

The uncorrected P values for the Chi-square tested frequency distributions of the four possible gamete types per locus pair are shown in Fig. 2. As parental and recombinant gametes were indistinguishable because the genotypes of the parents of the frogs crossed were unknown, insignificant P value deviations from zero do not necessarily imply reduced recombination. Insignificant P value would also have resulted from randomly derived excess of recombinant gametes and from uneven allele frequencies within the expected numbers of parental and recombinant gametes. When considered individually, the -log(p) values exceeding 1.30 were significant at the 0.05 level. After within-genome sequential Bonferroni correction for the 65 tests in the L genome and the 91 tests in the R genome, however, only four P values were significant. This indicates that the great majority of locus pairs were unlinked and freely recombined.

The four locus pairs showing significant linkage occurred in four different frogs (represented by four filled symbol types in Fig. 2) that all produced equilibrium offspring frequencies at their remaining locus pairs. The linkage was therefore rather a property of the loci than of the frogs involved. Unfortunately, replicate data was not obtained for the three locus pairs giving the most significant P values in this study, but the pair with strongest linkage, Re1CAGA10 + RlCA18 (L genome), was the same pair for which Arioli (2007 chap. 1) found no recombination. From the 40, 0, 0, 38 gamete frequency distribution in that and the 20, 0, 3, 23 gamete frequency distribution in the present study, it can be inferred that that Re1CAGA10 and RlCA18 are linked, i.e. situated closely together on the same chromosome. Ca1b6 + Ga1a19 (R genome) had the offspring type distribution 10, 33, 18, 8 and Rrid169 + Rrid059A (R genome) had 36, 16, 7, 27. These locus pairs thus appear weakly linked, but

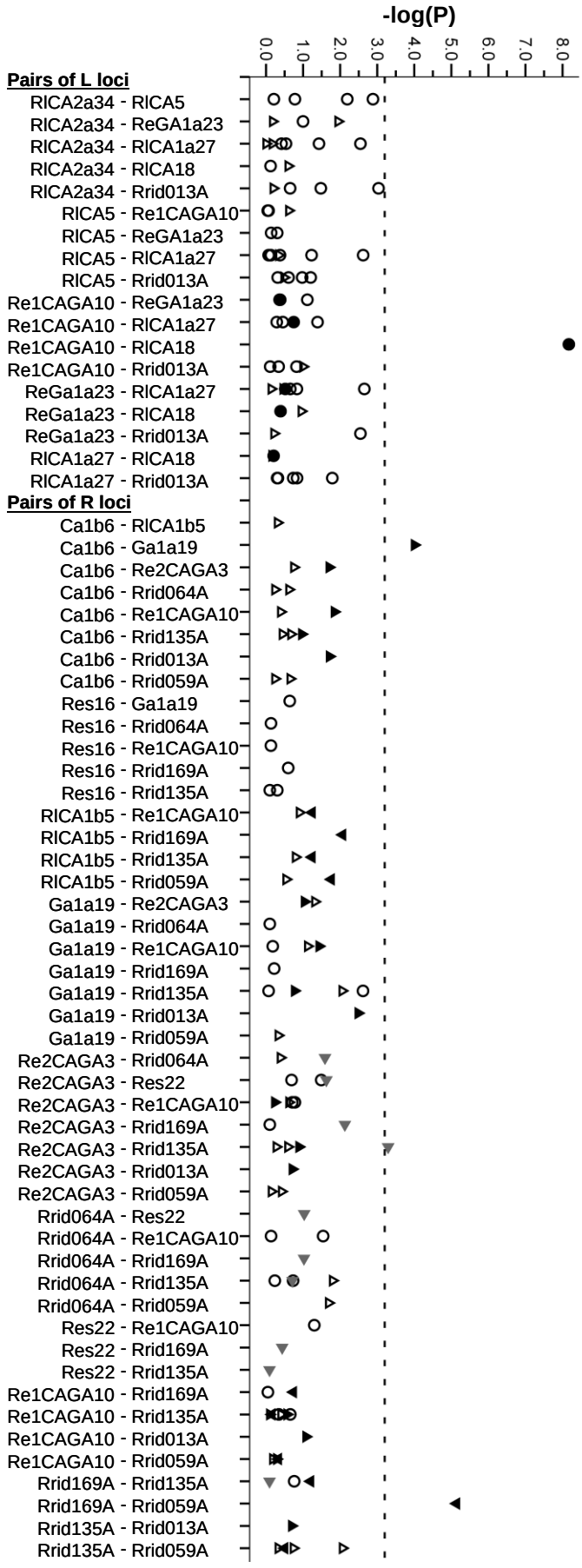

Figure 2. Linkage analysis of various locus combinations in crossing experiment with triploid *P. esculentus*. The symbols represent -$\log_{10}$ P values from Chi-square tests of the frequency distributions of the four potential (two parental and two recombinant) gamete types produced. Circles = males, triangles = females. Most individuals were heterozygous at several loci and therefore contributed data for several locus pairs. Each point left of the dashed line indicate a freely recombined locus pair in a frog. Points right of the dashed line indicate significant linkage at the 0.05 level after sequential Bonferroni-correction within each genome separately. Filled symbols (circles and triangles pointing right, left and down) identify all P values derived from the four individuals that each gave a significant P value. The female identified by grey triangles pointing down had a mutation at Re2GACA3 in her germ line which she passed on to some of her offspring.

replicate crossings would be needed to confirm linkage. Re2GAGA3 + Rrid135A appeared significantly linked in one female with gamete frequency distribution 12, 13, 20, 17, but unlinked in three other females. Overall, therefore, these two loci appear unlinked. Actually, a mutation happened in the germ line of this female so that some of her offspring had a new allele at locus Re2CAGA3. A rare allele at another locus confirmed that these offspring were indeed hers. The offspring with the new allele were excluded from the analyses involving Re2CAGA3, but when included by pooling the new and the lowest-frequency maternal allele, from which it most probably mutated, all four P values for locus pairs including Re2CAGA3 dropped substantially and the significant value became clearly non-significant.

Nearly significant P values appeared for several other locus pairs, but also here replicates raised the average for these loci to well above the 0.05 level, rendering no overall indication of linkage. Males and females did not have significantly different mean P values (male mean = 0.356, female mean = 0.285, t-test, $t_{154} = 1.606$, P = 0.110). Many species, probably including *P. esculentus* (Burt et al. 1991), have lower crossing-over rates in males than in females, but the present data set can neither confirm or disprove this for *P. esculentus*.

Natural populations

Triploids were found in all 55 ponds investigated, and both kinds (LLR and LRR) were found in 82% of the ponds. The proportion of LLR varied from 0-100% while that of LRR varied from 0-86% in the pond samples (Fig. 3). Of the 2296 frogs genotyped, only 0.2% were non-hybrid. These were 5 LL from two Swedish ponds. Multilocus linkage disequilibrium, measured as $\bar{r}d$ on a scale from zero to one, averaged 0.01 in the L genome and 0.11 in the R genome, indicating that both genomes were well recombined in the majority of the natural populations. Mean $\bar{r}_d$ in the R genome was, however, significantly higher than in the L genome (t-test, $t_{111} = -3.819$, $P < 0.001$).

Multilocus disequilibrium in the L genome showed no relation with the proportion of LLR individuals (linear regression: $F_{1,61} = 2.269$, P = 0.137, $r^2 = 0.036$). In contrast, multilocus disequilibrium in the R genome was negatively associated with the proportion of recombining LRR frogs among the R gamete-producing LR and LRR frogs (linear regression: $F_{1,54} = 9.034$, P = 0.004, $r^2 = 0.143$, slope = -0.214). These results were thus fully in accordance with the expectations. The slopes of the L and the R regressions were, however, not significantly different ($t_{115} = 1.440$, P = 0.153).

The multilocus linkage disequilibria ($\bar{r}_d$) in the L and the R genomes were not positively correlated within ponds (Fig. 4). In fact, they were significantly negatively

correlated (Pearson correlation: r_{55} = -0.374, P = 0.005); even excluding the L outliers far left and far right in Fig. 4. This indicates an absence of strong pond-specific effects affecting $\bar{r}_d$ in the L and R genome simultaneously.

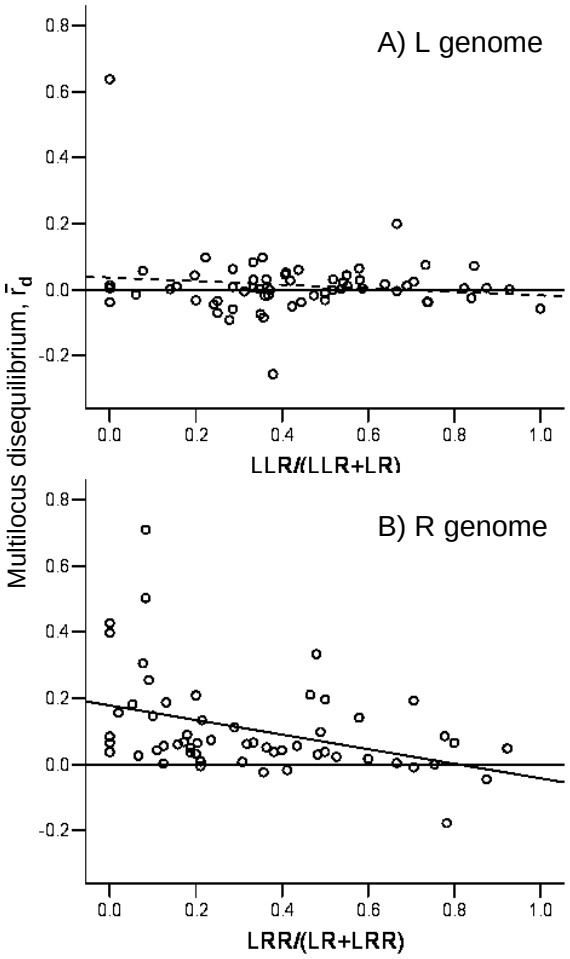

Figure 3. Multilocus linkage disequilibrium, $\bar{r}_d$, as a function of the proportion of frogs producing recombined gametes in 66 *P. esculentus* populations from Denmark and Sweden. a) $\bar{r}_d$ in the L genome vs. recombining LLR frogs of the total number of frogs propagating L genomes (LLR+LR). Linear regression line dashed because non-significant. b) $\bar{r}_d$ in the R genome vs. recombining LRR/total R-propagating frogs; regression significant. $\bar{r}_d$ is an index of association adjusted for unequal sample size.

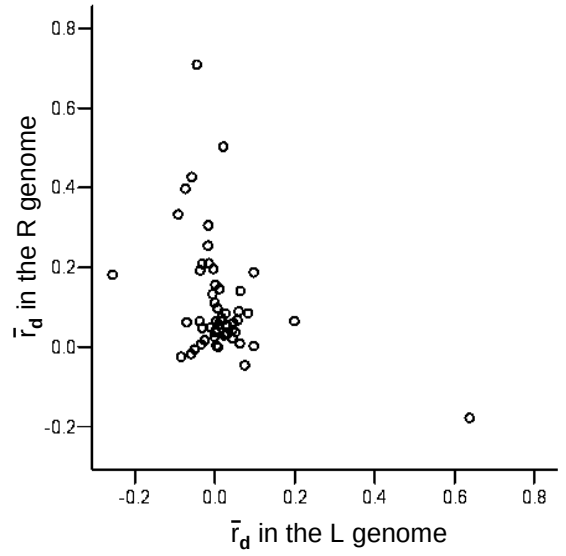

Figure 4. Multilocus linkage disequilibrium ($\bar{r}_d$) in the L vs. the R genome in 56 ponds.

There was no correlation between $\bar{r}_d$ and genetic diversity, measured as the expected heterozygosity summed over all loci (Pearson correlation for L and R data pooled: r_{119} = 0.013, P = 0.889). The significant difference in mean multilocus disequilibrium between the L and the R genome can therefore not be explained by lower polymorphism in the L specific microsatellite loci, but only by differences in recombination rates. The $\bar{r}_d$ values for the R genome showed also no correlation with the number of hemizygotes in the sample they were calculated from (Pearson correlation: r_{55} = 0.016, P = 0.904). The significant relation between LRR/(LR+LRR) and $\bar{r}_d$ in the R genome in Fig. 3b can therefore not be explained by exclusion of heterozygous loci in LRR frogs, but must be attributed to differences in recombination rates.

An analysis of pairwise $\bar{r}_d$ values showed that only two locus pair had $\bar{r}_d$ values differing significantly from the mean pairwise $\bar{r}_d$ of the remaining locus pairs in the same ponds (28 L and 63 R, paired t-tests with sequential Bonferroni correction within each genome separately). These two locus pairs (L loci Res20 + Re1CAGA10 and the R loci RlCA1b5 + Rrid064A) both had significantly lower $\bar{r}_d$ than the remaining loci. Thus, most locus pairs gave similar results within ponds and none gave elevated values suggesting linkage. In spite of the tight linkage in the crossing experiment, pairwise $\bar{r}_d$ for Re1CAGA10 + RlCA18 was not significantly different from the mean, even without Bonferroni correction (paired t-test: t_9 = 1.311, P = 0.222). The same applies to the two potentially linked locus pairs (Ca1b6 + Ga1a19: t_{10} = –0.654, P = 0.528; Rrid169 + Rrid059A: t_{22} = 1.261, P = 0.221). Therefore, these three (potentially) linked locus pairs were not excluded from the analyses of natural populations.

Global F_{IS} was very low in both the L and R genome, i.e. -0.007 and -0.008, respectively, indicating random mating within ponds. Global F_{ST} values were rather high, i.e. 0.4561 and 0.6156 in the L and R genome, respectively, indicating much genetic structure among ponds, which is in accordance with the expectations for a low-mobility animal. As a consequence of the low F_{IS}, F_{IT} was very similar to F_{ST} for both genomes.

Discussion

Recombination was demonstrated in all 30 frogs tested in the crossing experiment including both males and females of both LLR and LRR. As a consequence of such triploid-mediated recombination, natural populations were found to have low multilocus linkage disequilibria. In agreement with predictions from the asymmetrical propagation of L and R genomes in the all-hybrid populations, L genomes were generally fully recombined while R genomes were

recombined according to the proportion of LRR triploids. The unique all-hybrid populations of *P. esculentus* are thus functionally sexual; actually, they represent an obligate symbiosis of two independent, functionally sexual genomes: the L and the R genome. Below, we will first describe the genetic mechanisms underlying these results and then outline the evolutionary, conseptual and conservation-political implications for all-hybrid populations and hybridogenetic taxa.

Recombination in all-hybrid populations

In normal meiosis, the combined effects of random segregation of chromosomes and chromosomal crossing-over assure equal proportions of parental and recombinant gametes for most locus pairs. Reduced recombination rates due to physical linkage are, however, observed between loci situated so closely together on the same chromosome that there is small probability of crossing-over between them. A random sample of genetic markers for any kind of organism might thus include a small proportion of linked loci. *P. esculentus* has 13 chromosomes per L or R set (e.g. Koref-Santibanez and Günther 1980). The physical locations of our microsatellite loci on these chromosomes are unknown, but the results from the crossing experiment suggested linkage between three of the 65 locus pairs investigated. Loci Re1CAGA10 and RlCA18 showed strong linkage in a male crossed by us as well as in one crossed by Arioli (2007 chap. 1); thus it can be inferred that these two loci are situated close together. The apparent linkage of the two remaining locus pairs in this study was weaker and assessed in only one frog each, so that linkage should not be concluded without further verification. This discovery of one to three linked loci does not suggest variation in recombination rates among individuals, as the three frogs with apparently linked loci had full recombination at their remaining locus pairs investigated.

Selection took place in the crossing experiment, as dead and sick-looking tadpoles were not reared, and 10% of the offspring chosen for rearing eluded genotyping – mainly by dying. Only selection on the interaction of non-neutral loci linked to our markers could, however, have affected the recombination results. Any such interaction effects were reduced by crossing every parent to several mates of different genotypes. As no significant bias in the segregation at any single locus was detected, bias of locus combinations by selection is unlikely. Furthermore, selection on the interaction of linked non-neutral loci would most likely bias the results towards less recombination, so it would not undermine the conclusion of recombination.

Unlike linkage, linkage disequilibrium can arise between loci without physical associations. Linkage disequilibrium, measured as $\bar{r}_d$ in the natural populations, is the net result of generating and deteriorating forces. Linkage disequilibrium-generating forces include founder effect, migration, drift, inbreeding and selection on linked genes, called hitchhiking (Hedrick 2005). In clonal organisms, the entire genome hitchhikes with positively selected genes. The deteriorating force is recombination. Linkage disequilibrium is a negative linear function of recombination rate per generation, with half of the disequilibrium disappearing per generation at 100% recombination (Hedrick 2005). Provided that $\bar{r}_d$ is a good measure of linkage disequilibrium, that LRR/(LR+LRR) was a fair substitute for recombination frequency in the ponds, and that disequilibrium-generating forces did not depend on population structure (e.g. on LRR/(LR+LRR)), linear relationships were therefore expected in Fig. 3.

With low $\bar{r}_d$ irrespective of population structure in the L genome (Fig. 3a) and a negative relationship between $\bar{r}_d$ and recombining triploids (LRR/LR+LRR) in the R genome (Fig. 3b), the expectations outlined in the introduction were met. According to the model by Som and Reyer (2006a), L genomes spend 2/3 of their generations in LLR frogs and 1/3 in LR frogs, which means that they are recombined two out of three generations. The empirical data from the present study shows that this recombination rate of 2/3, whatever the type and strength of linkage disequilibrium-generating forces in the natural populations, is sufficient to reduce $\bar{r}_d$ values to around zero (mean $\bar{r}_d = 0.01$ on the scale from zero to one). For the R genome, no theoretical model is available. Before a reliable model can be made, more empirical data on the ratio of R and LR gametes produced by LR females and LR males is needed, as this ratio is important for population dynamics and has been shown to vary strongly between individuals and locations (Arioli 2007 chap. 1; Christiansen et al. 2005; Jakob 2007 chap. 5; Mikulícek and Kotlík 2001; Polls Pelaz 1994; Rybacki and Berger 2001; Tunner and Heppich-Tunner 1991). Central to such a model is also the question of why populations vary in structure. Although it is commonly accepted that population structure of *P. esculentus*, *P. lessonae* and/or *P. ridibundus* depend on ecological components (Holenweg Peter et al. 2002; Pagano et al. 2001; Plötner 2005), attempts to identify the ecological components determining population structure in Swedish all-hybrid populations were so far rather inconclusive (Jakob 2007 chap. 3). In the absence of theoretical models, it was not known what level of linkage disequilibrium to expect in the R genome of natural populations, but the present empirical data show that it is generally low (mean $\bar{r}_d = 0.11$), although the genetic signature of clonal reproduction was visible in certain populations with few LRR

frogs. In clonal populations of other organisms, $\bar{r}_d$ values have been found to be considerably higher than in the present study (e.g. Goyeau et al. 2007; Grundmann et al. 2008). Unfortunately, no thorough studies on multilocus disequilibrium in the *R. esculenta* LE or RE system have been conducted yet.

The variation not explained by the linear relations in Fig. 3 is expected to derive from three main sources. 1) Error on the estimate of $\bar{r}_d$ from a random sample of 17-86 (mean 35) individuals. 2) Error on the estimate of population structure, e.g. LRR/(LR+LRR), from the same random sample and between-pond-variation in the ratio of R gametes from LR frogs. If the proportion of R gametes made by LR frogs varies between ponds, this will add further noise. 3) Between-pond variation in the strength of the various disequilibrium-generating forces listed above. The combined effects of these three sources explain the rather large variation for the R genome in Fig. 3b. For the L genome, population structure (source 2) should have no relevance, however. Furthermore, if the recombination rate is so high that it always overpowers the local disequilibrium-generating forces (source 3), as seems to be the case in the L genome, variation comes only from the error on the estimate of $\bar{r}_d$ (source 1). This explains the relative low variation in Fig. 3a. Unfortunately, disequilibrium-generating forces are difficult to measure. The only disequilibrium-generating force, we could measure in this study was inbreeding. The low F_{IS} values obtained indicated random mating, so that inbreeding would have little effect on $\bar{r}_d$. We did, however, test for those pond-specific effects that affect the L and R genome similarly. The lack of a positive correlation between $\bar{r}_d$ in the L and R genome across ponds (Fig. 4) indicates that such forces were absent. In conclusion, the forces generating multilocus linkage disequilibrium in the natural populations could not be indentified, but between-pond variation in their strength and composition did not pose a problem in this study. On the contrary: the good match of observed with expected relations in Fig. 3a and b shows that $\bar{r}_d$ can be a useful tool in studies of recombination.

The extreme positive outlier in Fig. 3a calls for a different explanation than those given for residual variation. This explanation has to apply to the L genome only, as the high L $\bar{r}_d$ value was not matched by a high R value (Fig. 4). Notably, in this pond, a null allele was scored as a real L allele, because it did not pose a technical problem. As pairwise $\bar{r}_d$ values were elevated for all locus pairs in this pond, the null allele cannot account for its outlier status, however. Exclusion of the locus with the null allele reduced $\bar{r}_d$ to 0.29, i.e. the point remaind an outlier although less extreme. A better explanation for the high $\bar{r}_d$ value can be derived from the pond's extreme left position in the Figure. Although necessary for reproduction, LLR frogs were absent from our sample of 23 adults. Also notable, although not

exceptional for this pond, was that the population appeared small with few males, which are more often LLR than females. We could therefore speculate that the L genomes in the sampled frogs derived from very few LLR ancestors. A linkage disequilibrium in the L genome caused by such a bottleneck in LLR frogs would persist for several generations of recombination.

Evolutionary consequences

Triploids are not restricted to all-hybrid populations, but have been found in various population types in Germany (Günther 1975), Poland (Rybacki and Berger 2001) and France (Regnier and Neveu 1986). The ability to make diploid eggs giving rise to triploid individuals provides all these *P. esculentus* populations with genetic recombination and potential reproductive independence - two important steps in the direction of speciation. Where hybrids live sympatrically with parental species, they do not reproduce independently, however, but interbreed with the parental species. Here, recombination by triploids might be of little genetic importance to the hybrids, because they can be supplied with recombined genomes from the parental species. In contrast, the all-hybrid populations of Denmark and southern Sweden must rely on recombination in triploids only, as they are isolated from the nearest parental populations by sea or large stretches of uninhabited land, and non-hybrid LL and RR offspring only very rarely survive to sexual maturity (Christiansen et al. 2005; Jakob 2007 chap. 2 and the present study). Here, *P. esculentus* has truly accomplished the transition from a clonal, gamete-dependent hybrid to an independent, sexually reproducing evolutionary unit.

Although the all-hybrid populations have a combination of clonal and sexual reproduction, the low multilocus linkage disequilibrium values indicate that the loci of natural populations were well mixed. Selection should thus have the whole range of genetic combinations to work on, enabling beneficial, as well as harmful, mutations to be combined for fast adaption to changing environments (Fisher 1930) and for purging of deleterious mutations (Muller 1932). This hybridogenetic reproduction mode also ensures continuous genetic variation as a defense against fast evolving parasites (Red Queen hypothesis, Hamilton 1980), since the combination of recombined and clonal gametes result in unique individuals. The all-hybrid populations thus seem to have all the advantages of sexual reproduction, including a long-term evolutionary potential. The ability of fast adaption to changing environments might, however, be of more importance for the survival of *P. esculentus*, given that habitat loss and climate change increasingly threaten amphibians worldwide (Stuart et al. 2004).

It remains to be analyzed to what extent all-hybrid *P. esculentus* populations can also benefit from the clonal reproduction of diploids. In general, potential benefits of clonal reproduction include the possibility to save the costs of producing males and the ability to propagate favorable gene combinations (Otto and Gerstein 2006). In all-hybrid *P. esculentus* populations, the theoretical offspring sex ratio is only slightly female biased which is in agreement with the mean observed adult sex ratio in large surveys (Jakob 2007 chap. 2 and the present study; Som and Reyer 2006b). Thus, only a few percent of the cost of males might be saved. Recombination takes place after maximum one generation in the L genome (Som and Reyer 2006a) and after one to a few generations in the R genome, suggesting that favorable gene combinations are not be preserved for long, unless physically linked. Therefore, the benefit that all-hybrid populations of *P. esculentus* can potentially derive from the clonal component in their reproduction appears small - in contrast to cyclical parthenogens, such as aphids, rotifers, water fleas that have successfully combined the advantages of sexual and clonal reproduction (Innes and Singleton 2000).

With sexual reproduction, the death of newly formed non-hybrid LL and RR in the all-hybrid populations is intriguing, because it cannot be attributed to clonal propagation of the genomes, as in the LE system. In the LE system, RR die because recessive deleterious mutations have become fixed in the clonally propagated R genome of the diploid LR hybrids (Guex et al. 2002; Vorburger 2001a). These deleterious mutations were either acquired through Muller's ratchet or were already present at hemiclone formation (Vorburger 2001a). In all-hybrid EE populations, both genomes are regularly recombined in triploid individuals, the L when in LLR and the R when in LRR. Hence, fixation of deleterious mutations by Muller's ratchet is unlikely, yet fixation may still have occurred by other mechanisms, for example founder effect. Fixation and low genetic diversity is certainly observed at microsatellite loci (Arioli 2007 chap. 4 and the present study; Christiansen et al. 2005). Explanations for how genetic diversity became and remained this low in spite of the presence of parental species just south of the German and north of the Swedish all-hybrid populations are, however, lacking.

P. esculentus most closely resembles the Iberian minnow, *Squalius alburnoides* (also called *Leuciscus, Rutilus* and *Tropidophoxinellus*, reviewed by Alves et al. 2001) of other hybridogenetic taxa known: both hybrids often form mixed populations of di- and polyploid hybrids and one or both parental genotypes. All-hybrid di- and triploid populations are, however, not known from *S. alburnoides*. In stead, tetraploids occur in many *S. alburnoides* populations and, in special habitats, tetraploids can constitute 73% of the mixed populations.

These tetraploids have an even sex ratio, have normal meiosis, produce tetraploid offspring when mating with each other and appear to be reproductively isolated from other ploidy levels (Cunha et al. 2008). The discovery of these mainly tetraploid populations strongly suggests that meiotic hybridogenesis can act as a stepping stone to tetraploidization and ultimately to speciation. In *P. esculentus*, tetraploidy has so far only been found in very low frequencies in Swedish populations (Jakob 2007 chap. 2).

Given that recombination appears to be the rule in polyploid hybridogens and that polyploidy in hybridogenetic taxa appears to be more common than previously assumed, the prevailing view of hybridogens as clonally reproducing diploids may have to be changed. Should the discoveries of hybridogenetic breeding systems continue to increase, which is likely as more and more supposedly normal species are being genetically analyzed, this will also affect our perception of the importance of hybridization for speciation in animals.

Studies on hybrids are also relevant from a conservation point of view. Modern management concepts stress the importance of conserving "evolutionary significant units" (ESUs), i.e. populations representing significant adaptive variation; but how these units are to be identified, is strongly debated (reviewed by Crandall et al. 2000). Hybrids, for instance, are exempt from protection, because they do not seem to constitute independent evolutionary lineages (Kraus 1995). While this may be true for F_1 progeny from many interspecific matings, it is not true for parthenogenetic, gynogenetic and hybridogenetic taxa of hybrid origin, which are capable of self propagation (Kraus 1995; Ranker and Arft 1994). This, plus the finding that hybridogens like *P. esculentus* and *S. alburnoides* can form independent and sexually reproducing populations, makes these organisms evolutionary significant units and worthy of protection.

Acknowledgements

This study would not have been possible without the invaluable help from Lars Iversen, Ursina Tobler and Eline Embrechts with field work, and from Sandra Röthlisberger with laboratory work. Unpublished primers were provided by Trenton T. Garner and Hansjürg Hotz, whom we warmly thank for letting us publish them. Ecogenics (Zurich) helped testing primers in Danish and Swedish samples. Our acknowledgements also go to Josh Van Buskirk, Peter Wandeler, Lukas Keller, Christian Mayer and anonymous reviewers for valuable inputs to the methods and the manuscript. The study was supported by the Swiss National Science Foundation (grant no. 31-64004.00 to H.-U. Reyer). Permits for catching frogs, toe-clipping frogs and rearing tadpoles were obtained from the Danish and Swedish authorities (Skov- og

Naturstyrelsen SN 2001-441-0252, SNS-441-00047, Länsstyreslen I Skaane Län 522-10481-05, Djurskyddsmyndigheten M62-05, M71-06).

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BMC Evolutionary Biology 2009, vol 9, article 135

Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*)

Ditte G. Christiansen

Abstract

Background

Triploid individuals often play a key role in speciation by hybridization. An understanding of the gamete types (ploidy and genomic content) and stability of hybrid populations with triploid individuals is therefore of importance for exploring the role of hybridization in evolution. The all-hybrid populations of the edible frog, *Pelophylax esculentus*, are unique in their composition and genetic dynamics: Diploid (genotype LR) and triploid (LLR and LRR) hybrids depend on each other's different gamete contributions for successful reproduction and maintenance of the populations, as the parental genotypes *P. lessonae* (LL) and *P. ridibundus* (RR) are absent among adults. This study provides data and interpretations on gamete types and sex determination that are essential for understanding the function, evolutionary potential and threats of this intriguing system.

Results

Dissection of metamorphs from a crossing experiment confirmed that sex determination is an XX-XY system with the Y confined to the L genome. From microsatellite analysis of parents and offspring from the crossings, gamete frequencies could be deduced: Triploids of both sexes mostly made haploid gametes with the genome they had in double dose, however LLR females also made approximately 10% LL gametes by automixis. LR frogs showed much variation in their gamete production. In LRR-rich populations, their LR sperm production was sufficiently high (22%) to explain the observed proportion of LRR males, the formation of which has not previously been understood. A model was constructed to calculate equilibrium

genotype proportions for different population types on the basis of the gamete proportions found. These equilibria agreed well with empirical literature data.

Conclusions

If population differentiation with respect to genotype proportions is really driven by gamete patterns, as strongly suggested by the present study, all-hybrid populations constitute not one, but several intrinsically different breeding systems. Tetraploidization could occur if the survival or fertility of both males and females increased. Whether introduction of hybrid or parental species individuals would threaten the all-hybrid populations cannot be predicted without further knowledge on the mechanisms behind non-hybrid inviability, but at least R genomes with Y factor are predicted to be invasive, if introduced, and could bring the populations to collapse.

Background

Hybridization is a major creative force in evolution, especially in plants [1], but also of importance in animals [2-4]. Hybridization frequently leads to polyploidy, because the combination of two different genomes often disrupts meiosis and, hence, results in unreduced, diploid gametes [5, 6]. The larger the genetic distance between the parental species, the higher the proportion of polyploid hybrids [7]. Tetraploidy can be very advantageous to hybrid taxa, as it can both restore normal meiosis and establish a reproductive barrier to the parental species – two key elements in hybrid speciation.

Although tetraploids can arise directly from diploid progenitors producing unreduced gametes, tetraploids are often formed by an intermediate triploid step: It has been estimated that 30% of the tetraploidization events in hybrid flowering plants are mediated by triploids which make diploid or triploid gametes [5]. Examples of triploid-mediated tetraploidization are also known from animals [8, 9]. Studies on gamete types and stability of hybrid populations with triploid individuals are therefore of importance for a broader understanding of speciation by hybridization. The present study focuses on all-hybrid populations of the edible frog, *Pelophylax esculentus*, where triploids demonstrate an alternative way of providing genetic recombination and reproductive independence than by mediating tetraploidization.

Pelophylax esculentus (genus *Rana* until [10]) is a hybrid between the pool frog, *P. lessonae* (genome LL) and the lake frog *P. ridibundus* (genome RR). It is widespread in

Europe; often as diploid LR that is dependent on gametes from one or the other parental species. In the LE system, i.e. the *lessonae-esculentus* system, LR frogs exclude the L genome during gametogenesis and make exclusively clonal R gametes (Fig 1a; hemiclonal reproduction, hybridogenesis). They must therefore mate with *P. lessonae* to produce new hybrids (reviewed by e.g. [11]). Inter-hybrid matings result in RR offspring that typically die before sexual maturity, because they are homozygous for deleterious mutations in the clonally propagated genome ([12, 13] and references in the former). A reverse form of this breeding system exist as the *ridibundus-esculentus* system, or RE system for short (reviewed by [11, 14]). Here LR predominantly produces L gametes and must therefore mate with *P. ridibundus* to form new hybrids (Fig 1b).

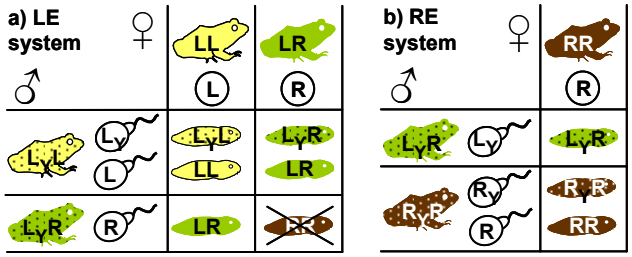

Fig 1. Schematic drawings of the a) LE (*lessonae-esculentus*) and b) RE (*ridibundus-esculentus*) breeding systems with adults (frog silhouettes), their gametes (eggs and sperm) and the resulting offspring (tadpole silhouettes). Yellow (LL) = *P. lessonae*, green (LR) = *P. esculentus*, brown (RR) = *P. ridibundus*. The Y subscript denotes the male-determining Y factor; all genomes (letters) without subscript carry an X factor. In the LE system, the confinement of the Y factor to the L genome produces female excess among the hybrid offspring. RR offspring typically die before sexual maturity. In the RE system, *P. esculentus* is all-male in diploid populations.

Although the diploid LE and RE systems are nearly mirror images of each other at the genomic level, an asymmetry in the sex determination system results in very different sex ratios. Sex determination in *P. esculentus* is a genetic XX-XY system with almost no differentiation between the sex-determining chromosomes [15]. For size-related behavioural reasons, most primary hybridizations take place between *P. lessonae* males and *P. ridibundus* females, and thus the Y factor becomes confined to the L genome in hybrids [16]. In other words, the hybrids' L genomes can have an X or a Y factor while their R genomes only have an X factor. In the LE system, this asymmetry leads to an expected (Fig 1a; [17]) and observed (~60%, [16]) female bias, while in diploid RE populations, all hybrids are males (Fig 1b; [18]).

However, the LE and RE systems are not always regular diploid systems as described above. In some areas, many different combinations of the parental species (LL and RR), diploids hybrids (LR) and triploid hybrids (LLR and LRR) can be found (e.g. [19]) and little

is known about how these populations function. Sex determination need also not always be as described above [16].

Triploid hybrids, LLR and LRR, enable *P. esculentus* populations to persist without the parental species. All-hybrid populations have been reported from many areas, but in most cases there is insufficient evidence for their isolation from the parental species and long-term stability [19-23]. However, in a large area covering Southern Sweden, Denmark and Northern Germany, intensive studies have documented the absence of adult *P. lessonae* and *P. ridibundus* [24-26]. These studies also showed that, in general, triploid frogs make haploid gametes with the genome they have in double dose, i.e. LLR frogs of both sexes make L gametes, and LRR frogs, which are mainly females, make R gametes. As to the diploid frogs, LR males make R gametes, like in the LE system, and LR females make both R and LR eggs, the latter giving rise to triploids upon fertilization by haploid sperm [24-26]. As LLR frogs have genetic recombination between their two L's and the LRR frogs recombine their two R's, the all-hybrid populations are overall functionally sexual [27]. The Y factor is assumed to be confined to the L genome as is the norm in the LE and RE systems, but this has not been investigated. Non-hybrid LL and RR offspring, are formed in every generation, but disappear from natural ponds during larval development [25]. The all-hybrid populations thus thrive in spite of a considerable hybrid load, that results from the wasteful production of non-hybrids plus inviable mixed genotypes arising by gametogenetic errors [24].

Although these findings give us a rough idea of how the all-hybrid populations maintain themselves, there are at least three gaps in our knowledge. First, the gamete table in Fig 2 is incomplete: Sample sizes have till now been insufficient for an estimation of the mean proportions of LR and R eggs laid by LR females, as individual differences are large. The same applies to estimations of rare gametes produced by the other genotypes. For example, LL eggs [26], LL sperm ([21] and references therein) and LR sperm [28] have been reported from all-hybrid populations, but it is not known how frequent they are and whether they have importance for the dynamics of the populations. Second, it is not known whether the Y factor is confined to the L genome as in the LR and RE systems. Filling the first and second gaps should provide a solution to the riddle of LRR males. Our present understanding of the gamete pattern and sex determination (Fig 2) does not allow for the formation of LRR males. Yet, some ponds have persistent high proportions of LRR males [24, 26]. The presence of these LRR males could indicate the presence of R genomes with Y factor in these populations. Alternatively, LRR-males could be formed from diploid L_yR sperm (not in Fig 2) fertilizing R eggs, or by RR eggs (not in Fig 2) fertilized by L_y sperm. Third, natural ponds

are very heterogeneous in their relative proportions of diploid and triploid males and females [24, 26]. This could be due to variation in gametogenetic patterns between ponds, to differential environmental selection on the various genotypes, or both.

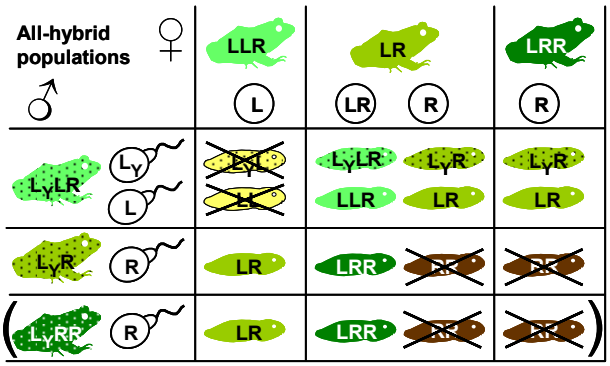

Fig 2. Conventional pattern of gametogenesis and reproduction in all-hybrid populations of diploid (LR) and triploid (LLR and LRR) *P. esculentus*. It is assumed that the dominant male-determining Y factor only occurs in males' L genomes. All chromosome sets (letters) without subscript in the figure have an X factor. Non-hybrid offspring (LL and RR) do not survive to sexual maturity and are therefore crossed out. Note that male LRR offspring (L_yRR; dark green with dots) are not formed by this pattern of gametogenesis, although they sometimes occur in natural populations. The LRR male and his offspring are therefore in parenthesis.

To quantify gamete proportions, explain the formation of LRR males and investigate whether gamete production varies between ponds, I performed a crossing experiment with 68 *P. esculentus* frogs from 11 Scandinavian ponds with various genotype compositions. Gamete type proportions were deduced from multilocus microsatellite analysis of both parents and offspring, whereas the offspring sex was determined by dissection shortly after metamorphosis.

Because even basic information about the all-hybrid populations is lacking, it is also unknown what sex- and genotype ratios to expect in natural all-hybrid populations at equilibrium. For addressing this question, I made a simple deterministic model that calculates the genotype proportions for 70 successive generations of an all-hybrid population under various scenarios of genotype ratios in the start population, gamete production patterns and genotype-specific survival. This model was used to find stable equilibrium population compositions with and without L-confined Y factors, to explore mechanisms that increase tetraploidy and to evaluate if introduction of parental species could pose a threat to these unique all-hybrid populations.

Methods

Source ponds

The *P. esculentus* crossed came from 10 ponds in Scania, Southern Sweden [26], and "Alsønderup", Northern Sealand, Denmark [24]. These and a few more ponds (except By011 and Road) had their genotype proportions monitored for years [24, 26]. As opposed to most, i.e. "normal" populations, where LRR-males are rare or absent, ponds 089, 138 and

Alsønderup had strikingly high proportions of LRR males (20%, 28% and 50% of the males, respectively), and were therefore called "LRR-rich" populations. Of the remaining populations, ponds 001 and 102 had the highest proportions of LLR frogs (69% and 51%, respectively) and were called LLR-rich. Pond 011 and supposedly also the 60m distant by011 had the highest proportion of LR frogs (58%) and were called LR-rich.

Crossing, rearing and data collection

Crossing and tadpole rearing took place at Stensoffa Field Station in Scania, Sweden, 2006; see Christiansen and Reyer [27] for more details than provided here. A total of 269 frogs were caught and genotyped (see below), from which a subset was selected for the crossings. Among triploids, individuals with high heterozygosity at the two L or two R genomes were preferred (exemplified in Additional file 1). The selected frogs were hormone-treated and crossed artificially [29]. Six "full" crosses were made where all frogs were crossed to at least one partner of each of the genotypes LLR, LR and LRR (half-sib design; Table 1). This basic design was extended with an additional LR male and "extra" males and females whose numbers and genotypes varied among crosses. The complete design of each cross can be deduced from Additional file 2 and Additional file 3.

Table 1: Design of the full crosses (crosses 2, 3, 4, 5, 6 and 16)

	LLR female	LR female	LRR female	(extra female)
LLR male	sibship A	sibship B	sibship C	(sibship I)
LR male (non-LRR-rich)	sibship D	sibship E	sibship F	(sibship II)
LR male (LRR-rich)	sibship G	sibship H	sibship J	(sibship III)
LRR male	sibship K	sibship L	sibship M	(sibship IV)
(extra male 1)	(sibship N)	(sibship P)	(sibship Q)	(sibship V)
(extra male 2)	(sibship R)	(sibship S)	(sibship T)	(sibship VI)

As LR was the only genotype easily obtained from all population types, only LR frogs were used to test for population type-specific differences. For LR males, differences in the gamete pattern could be expected between normal and LRR-rich populations, as two of the three paths to LRR male formation involve LR males (R genomes with Y factor and LR sperm). Therefore, one LR male from each of these two population types (normal and LRR-rich) were included in all the full crosses. LR females were thought to be the genotype with the most individual variation in gamete proportions, requiring the largest sample size. To greatly enhance the sample size of LR females without making the full crosses too large, four "LR female crosses" (Table 2) were made in addition to the six full crosses. In each of these LR female crosses, three LR females were crossed with two random males, whose genotype

varied among the four crosses. Overall, the full and LR female crosses were balanced with respect to source pond type of the LR females (LLR-, LR- and LRR-rich), as the mean proportion of diploid versus haploid eggs might be expected to be lower in diploid-rich (LR-rich) than in triploid-rich (LLR-rich and LRR-rich) populations.

Table 2: Design of the LR female crosses (crosses 13, 14, 15 and 17)

	LR female, LLR-rich	LR female, LR-rich	LR female, LRR-rich
Random male 1	sibship A	sibship B	sibship C
Random male 2	sibship D	sibship E	sibship F

When two days old and still round, the eggs from at least one sibship per female (all A, B, C and I sibships; see Tables 1 and 2), were, if size dimorphism was visible, sorted into two or three size classes, as determined by eye, photographed and counted (Fig 3). These size classes, as well as the sibships, were kept in separate tubs throughout the rearing to be able to investigate whether size corresponded to ploidy. In sibships with correspondence, the proportions of different gametes produced by the female were estimated from the sorted and counted eggs, rather than from the subset of DNA tested individuals.

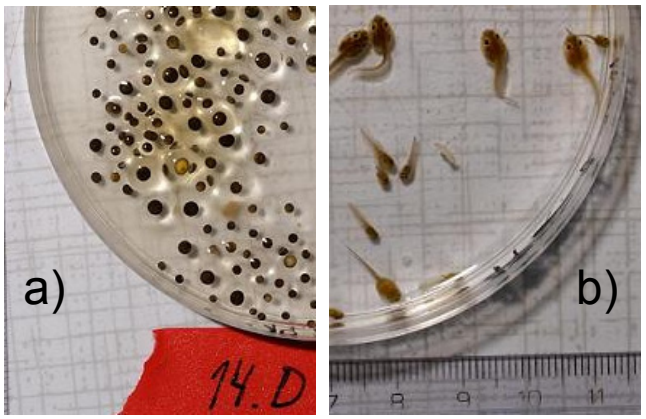

Fig 3. Extreme size dimorphism between a) large diploid and small haploid eggs and b) the resulting triploid and diploid tadpoles from female F11. Scale in cm. (The tadpoles were only temporarily in the petri dish for photographing). Photos: Lars Iversen.

Upon reaching the free-swimming feeding stage, the offspring from the LR female crosses were ended and at least 10 healthy-looking tadpoles (if available) from each egg size class were sampled for genotyping. From the full crosses, 15 healthy-looking tadpoles (if available) from each tub were picked and reared in 40-litres outdoors tubs for later sex determination. After reaching metamorphosis, they were transferred to smaller containers indoors. Seven to ten days after tail resorption, they were killed or anesthetized in an MS-222 solution (A5040, Sigma) and had their neck cut. A tissue sample was taken for genotyping and the sex was determined by dissection at 10 x magnification (Fig 4). Both the left and the right gonads were inspected.

Large tadpoles and metamorphs that died during rearing were also sexed and genotyped if not too rotten. In total, 1628 tadpoles from the six full crosses were selected for rearing, 1463 offspring (90%) were genotyped successfully and 1417 (87%) were sexed successfully. From the LR female crosses, 266 of the 267 samples were genotyped successfully. Permits for crossing frogs and rearing tadpoles were obtained from the Danish and Swedish authorities (Skov- og Naturstyrelsen SN 2001-441-0252, SNS-441-00047, Länsstyreslen I Skåne Län 522-10481-05, Djurskyddsmyndigheten M62-05, M71-06).

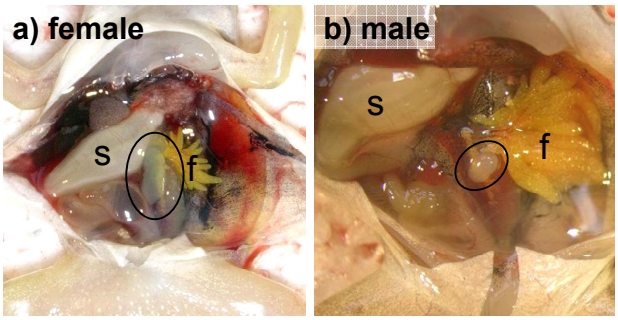

Fig 4. Encircled a) ovary, b) testis in *P. esculentus* 7-10 days after completed metamorphosis. Ovaries are large, long, flat and soft while testes are small, round and hard; the male is here shown with the higher magnification. The sacrificed froglets were fixed with the head upwards and cut open; the stomach (s, white) was pushed left and the fat body (f, bright yellow) right to reveal the left gonad (white) attached close to the spinal cord. Photos by the author.

DNA analysis

The genomic composition of adults, their offspring and finally their gametes was deduced using microsatellite analysis, as in studies of the di- and polyploid *Ambystoma* salamander complex [30] and other studies of the *P. esculentus* complex [24-26]. Examples are provided in Additional file 1.

The ethanol-stored tissue samples from adults and offspring were DNA-extracted with Qiagen BioSprint 96 DNA Blood Kit. All samples were then subjected to two multiplex PCRs with nine primer pairs each and the colour-labelled PCR products were visualized on an ABI 3730 Avant capillary sequencer. For laboratory protocols, see Christiansen and Reyer [27]. However, instead of forward primer Ga1a19, forward primer Ga1a19redisigned with sequence GCA CAC TAT TTC TGC TGT ATT GC was used. This redesigned primer amplifies 97 base pairs more than the original one and was actually also used instead of the original one in previous studies [25-27] without the knowledge of the authors.

The two multiplex PCRs amplified a total of 13 loci in the L genome and 13 loci in the R genome. Of these, 8 L loci and 12 R loci were polymorphic in the parents with 2-5 alleles each. All alleles were specific to either the L or the R genome. Four of the primer pairs amplified both L and R specific alleles and showed dosage effect, i.e. they could be used to distinguish between LLLR, LLR, LR, LRR and LRRR by the relative intensity of the L and R genome-specific alleles amplified [31]. The genome composition of hybrid adults and

offspring was thus determined independently four times and furthermore checked for agreement with the remaining, less informative, loci. Also the ploidy of non-hybrid offspring (LLL, LL, RR and RRR) could usually be determined by dosage effect at one or more heterozygous loci. Samples where loci after repeated analysis disagreed on the genotype were classified as "mixed genotypes", as it is very often not possible to deduce whether such mixed genotypes arose through aneuploidy, null alleles, mutations, recombination between L and R, or incomplete allele specificity (see Additional file 1 for examples). However, for most purposes, they were assigned to the genotype indicated by the majority of the loci.

LLRR individuals with two L and two R genomes that are pairwise indistinguishable with the 18 primer pairs used, will be misclassified as LR by using the above method. However, as only 8.8% of the LLR and 17.0% of the LRR frogs of the 269 frogs caught for the crossings had indistinguishable L and R genomes, respectively, the probability of misclassifying wild-caught LLRR frogs was only 0.088*0.17 = 0.015. As adult LLRR frogs were very rare in the source ponds (12/3792 = 0.3% captures in Sweden, Arioli and Jakob personal communication, and 0/46 in Alsønderup as determined by erythrocytes, own unpublished data), the probability that one of the 19 LR females crossed were actually a misclassified LLRR female is only 0.0009. LR males were not misclassified, as all LR males crossed had their diploid genotype confirmed by inspection of erythrocytes, which are larger in tetraploids than in diploids [26]. Also in the offspring, misclassification of LLRR as LR was minimal, as all parents crossed to each other differed in their allele composition.

Statistics

Population-type specific differences in gametes of LR males and of LR females were tested with non-parametric tests (in SPSS version 13.0 for Windows, SPSS Inc.), because the gamete data violated the assumptions of parametric tests.

Model

A model (Additional file 4) was constructed which basically is a quantitative extension of Fig 2 in Excel (Microsoft ® Office Excel 2003 (11.8220.8221) SP3, Microsoft Corporation).

The model requires the following input parameters:

a) The initial proportions (or numbers) of the different genotypes among adults in generation zero (LLL, LL, LLLR, LLR, LR, LLRR, LRR, LRRR, RR and RRR females and males). The Y factor can be present in the L genome, R genome, or both.

b) The proportions of the different gametes made by each genotype (LL, L, LR, R, RR eggs and sperm). The sum of the proportions determines the relative reproductive output, which can be varied to simulate differences in fertility, fecundity and mating success.
c) The relative survival of each offspring genotype (0-1).
d) The proportion of the previous generation that survives into the next (0-1).

Based on the input parameters, the model does the following calculations:

1) The adult genotype proportions (a) are standardized to 100% females and 100% males, as females and males contribute equally to all offspring.
2) The adult genotype proportions (step 1) are multiplied with their corresponding gamete proportions (b) to obtain egg and sperm genotype proportions in the population.
3) The egg and sperm genotype proportions (step 2) are multiplied to obtain offspring genotype proportions.
4) The offspring proportions (step 3) are multiplied with the genotype-specific offspring survival (c) to get the new generation of adults.
5) The new generation of adults (step 4) is standardized to 100%.
6) The proportion that survived from the previous generation (d) is added to one minus this proportion of the new generation (step 5).
7) Steps 1-6 are performed 70 times to obtain 70 successive generations.
8) A graph is produced that shows every generation's adult proportions (step 6) so that it can be assessed if equilibrium is reached at generation 70.
9) Another graph shows the proportion of non-hybrids among offspring in each generation (step 3), which in all-hybrid populations do not survive.

The model thus assumes euploid (not mixed) genotypes, random mating between genotypes, and an infinitely large population with no stochasticity (deterministic model). It also assumes that all genotype-specific selection takes place before reproduction.

As default, the populations were started (a) with equal proportions of LLR, LR and LRR males and females. The gamete proportions (b) of LLR, LR and LRR frogs were based on the results from the crossings. LLRR were assumed to make LR gametes by normal meiosis, since none were available for crossing. As the gametogenesis in asymmetric tetraploids (LLLR and LRRR) and triploid non-hybrids (LLL and RRR) is potentially

problematic and unknown, these genotypes were assumed to be sterile in the model (reproductive output = 0). Their assumed sterility should not have affected the results, since their abundances were always only fractions of percents (see results). For all the remaining genotypes, the gamete proportions added up to one, i.e. the reproductive output was set to one, unless otherwise stated. The relative survival (c) was set to one for all the hybrid genotypes and to zero for the non-hybrid genotypes, unless otherwise stated. The generation overlap (d) was set to 0.3 according to estimates from natural populations [26]. Populations where no genotype proportions changed over 70 generations when rounded to the nearest whole percent were recorded as in equilibrium.

Results

Crossing experiment

Crossing results were obtained from 32 males and 33 females in the crossings; one LR male, one LRR male and one LR female gave no offspring. In most cases, the maternal and paternal contribution to offspring could be determined by non-shared alleles. Where parentage was unclear for gametes with low frequencies, the possible frequency interval is indicated; for common gametes, doubtful cases were simply excluded. 3.6% of the offspring from the full crosses (raised to metamorphosis) and 19.5% from the LR female crosses (only raised to the beginning of the feeding stage) had spontaneous mixed genotypes, with one to half of the loci disagreeing on the genotype (see Additional file 1 for examples). The full results (Additional file 2 and Additional file 3) are summarized in Table 3. In this table, the results from each parent are weighed equally, irrespective of the number of gametes analyzed.

Table 3. Mean gamete proportions in different *P. esculentus* genotypes (and population types)

Sex	Genotype (pop. type)	n	LL%	L %	LR %	R %	RR%	LLR%
Male	LLR (all)	12	0.0-0.2	100				
Male	LR (normal)	7		16.3	0.3-0.6	83	0.3	
Male	LR (LRR-rich)	7			22.1	77.9		
Male	LRR (all)	6				99.7	0.3	
Female	LLR (all)	7	11.1-11.5	88.9				
Female	LR (LLR-rich)	6			54	45.7	0.3	
Female	LR (LR-rich)	6			91.5	8.3		0.2
Female	LR (LRR-rich)	6			99.7	0.3		
Female	LR (all) *	18			81.7	18.1	0.1	0.1
Female	LRR (all)	8				99.8	0.2	

* NB. LR females are listed twice in this table: first under LLR-rich, LR-rich or LRR-rich and then under “all”.

As expected, LLR frogs of both sexes made mainly or exclusively L gametes while LRR frogs made almost exclusively R gametes (Table 3, Additional file 2, Additional file 3). The exception was LLR females that made 11.1-11.5% LL eggs. This was mainly due to one female with 42% LL eggs, but two other females contributed considerable proportions too. LR frogs of both sexes also showed large variation in their gamete proportions. In addition to the expected R sperm, some LR males produced a surprising variety of other sperm types (L, LR and even one RR sperm). LR females produced on average 81.7% LR and 18.1% R eggs.

Six (= 2.2%) of the 269 adults caught for the crosses had slightly mixed genotypes, meaning that only one locus disagreed with the others. As these were all fertile without exhibiting unusual gamete patterns, they were included in the data presented above. Two of them (male M3, Additional file 2 and female F6, Additional file 3) were LLR frogs with a null allele in their R genome which was not passed on to the offspring. Two other adults crossed (M9 and F33) were triploids with an apparently substituted allele, i.e. an R allele in the L genome or vice versa, in one of the genomes they had in double dose. It is not clear whether these apparently substituted alleles had resulted from recombination between L and R, or from length mutation of the microsatellite loci in question. The L and R-specific alleles differed by 6 (at Res16) and approximately 15 (at RlCA1b5) base pairs in the two frogs, respectively. These frogs passed on the apparently substituted allele like a normal allele to approximately half of their offspring. The last two mixed genotypes (M15 and F23) were LR with a normal and a weakly amplifying allele at one locus. Re-extraction did not change this strange amplification pattern. Most offspring had the normal allele; very few or perhaps none inherited the weakly amplifying allele, but it was not weak in the offspring.

Six triploid parents each produced from one to 21 LL or RR gametes. These gametes were compared to the LL or RR parts of the triploid parents at the two to five loci where the parents were heterozygous. A total of 12 LL eggs contained both parental alleles at all the loci. In contrast, 22 LL eggs, 1 RR egg and 1 RR sperm contained both parental alleles at some of the loci, but only one of the parental alleles (probably in two copies) at other loci (see Additional file 1 for examples). Finally, 1 LL egg contained only one of the two parental alleles at all loci. This reduced heterozygosity indicates that most or all of these gametes contained not just the remaining LL or RR after exclusion of the rarer genome in the triploid parent (apomixis), but had gone through either duplication and meiosis or meiosis and fusion (automixis).

In 18 of the 33 females crossed, the size sorting of the eggs from the first sibship reflected the egg ploidy (left part of Additional file 3; the 15 females without size data either

laid just one size of eggs, or the attempted egg sorting did not reflect differences in genomic content). However, in 10-11 of the 18 cases where small and large eggs differed in genomic content, the less frequent size class was dominated by inviable and/or badly mixed genotypes. The latter, being diploid at some loci and triploid at other loci, were assumed not to contribute to future generations, as such genotypes are very rare among adults. They were therefore excluded from the gamete frequencies (Table 3 and right part of Additional file 3).

With respect to sex determination, L sperm from LLR males (with two L genomes) gave approximately equal proportions of sons (mean 47.3% ± SD 17.0) and daughters (52.7% ± 17.0). L and LR sperm from LR males (with only one L genome) gave almost only sons (100.0 ± 0.0 and 94.6% ± 9.3, respectively), while R sperm, irrespective of the parental genotype, rarely produced sons (5.9% ± 11.2). In contrast, no patterns in female genotype and offspring sex were observed (data not shown). This confirms that the male determines the offspring sex and that the Y factor is confined to the L genome, in both normal and LRR-rich populations. Although a number of apparent sons from R sperm were found, they derived from many different males and were always vastly outnumbered by sisters. They do therefore not indicate that their fathers had an R genome with Y factor. Alternative explanations for the occurrence of these unexpected sons are contemplated in the discussion.

LR males differed in their second-most common sperm type: those from normal populations produced 16.3% L sperm while those from LRR-rich populations made 22.1% LR sperm. This difference might explain the absence and presence of LRR males in normal and LRR-rich populations, respectively, as LR sperm, unlike other sperm types, frequently lead to the formation of LRR males. Due to large individual variation and small sample size, the difference between the LR sperm patterns in normal and the LRR-rich populations was, however, not significant (Mann-Witney U Tests, two-tailed: L $sperm_{7,7}$ = 14.0, P = 0.62; LR $sperm_{7,7}$ = 16.5, P = 0.20). The mean proportion of haploid R eggs from LR females differed much but insignificantly between LLR- (45.7%) LR-(8.3%) and LRR-rich (0.3%) populations (Kruskal-Wallis Test, two-tailed: $Chi\text{-}square_2$ = 5.5, P = 0.64). Contrary to the expectations, there was no trend of R eggs being more frequent in LR-rich populations than triploid-rich populations, as LLR- and LRR-rich populations differed the most (Table 3).

Model

In the model, input populations that were not inviable from the beginning proceeded to a stable equilibrium. This equilibrium depended on only two of the four input parameters mentioned in the methods, namely gamete production (b) and relative offspring survival (c).

The two remaining parameters, initial adult population (a) and generation overlap (d) only affected the number of generations required for reaching equilibrium.

With non-hybrids inviable, the normal all-hybrid population quickly stabilized at 35% males and 62% females (Fig 5a). LRR males and the various tetraploid genotypes each constituted less than one percent. At this equilibrium, 32% off all offspring were non-hybrid and therefore wasted. With the alternative sperm pattern of LR males from LRR-rich populations, the model population became more LRR-rich indeed (Fig 5b). The 22% LR sperm from these males made LRR the most frequent genotype in both males and females. It

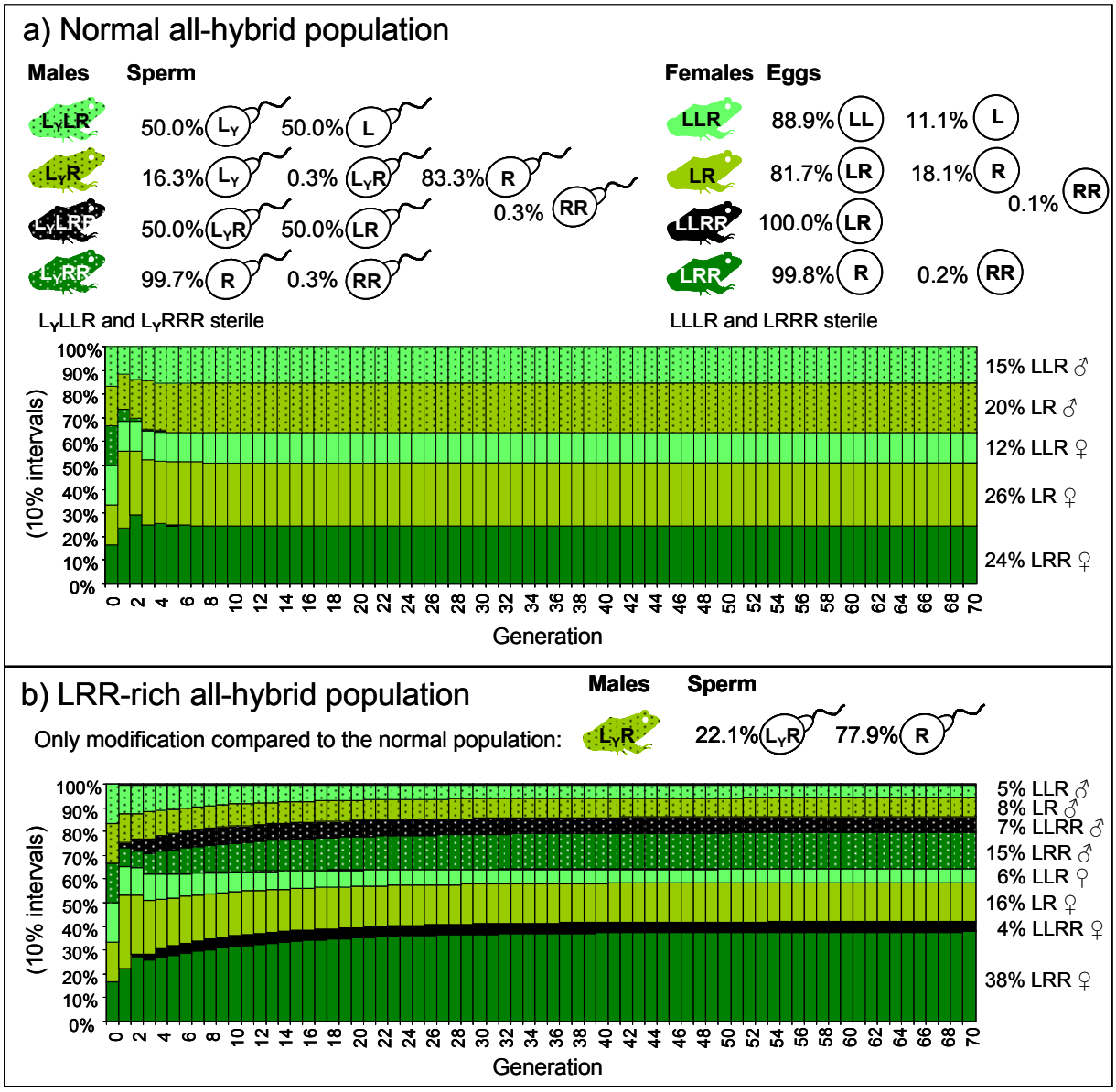

Fig 5. Input gamete data, population development and population equilibrium for a) a normal and b) an LRR-rich all-hybrid population of *P. esculentus*. The only difference is the gamete production of LR males. Both populations were started with equal proportions of LLR, LR and LRR males and females (generation 0), however any start population, if viable, produced the same equilibrium population. The survival of all the hybrid genotypes was set to one while that of non-hybrids (LLL, LL, RR, RRR) was zero.

also caused the presence of 11% LLRR, including both sexes. The hybrid load in the system increased, as 40% of the offspring were non-hybrid.

With respect to the proportion of R eggs in eggs from LR females, the 46%, 8% and 0.3% found in LLR- LR- and LRR-rich populations, respectively, could not create LLR- LR- and LRR-rich populations, respectively (data not shown). On the contrary, 46% R gave fewer LLR and more LR than 8% R. The proportion of LR adults generally increased with R egg proportion, however, R egg proportion had little effect on the genotype proportions as long as it stayed below approximately 50% with the sperm pattern of the normal populations and 80% with the sperm pattern of the LRR-rich populations. Thus, only higher than observed R egg proportions could potentially form LR-rich populations; low proportions do not yield LLR- or LRR-rich populations. The mean of 18% R eggs from LR females was therefore used in the previous and following model runs.

Although no R genomes with Y factor were found in the crossing experiment, the effect of introducing males with R genomes with Y factor into the all-hybrid model population with normal gametogenesis was also tested. The result was that they replaced all L genomes with Y factor (Fig 6). This is because LR males can propagate the Y factor much better if it is situated in the R genome than if it is in the L genome, as LR males make mostly R gametes. The resulting population lost its female bias and LRR became the prevailing genotype – especially in males. As a consequence of the R prevalence, 51% of the offspring were inviable RR non-hybrids. Also with the LRR-rich sperm pattern did R_Y outcompete L_Y (data not shown).

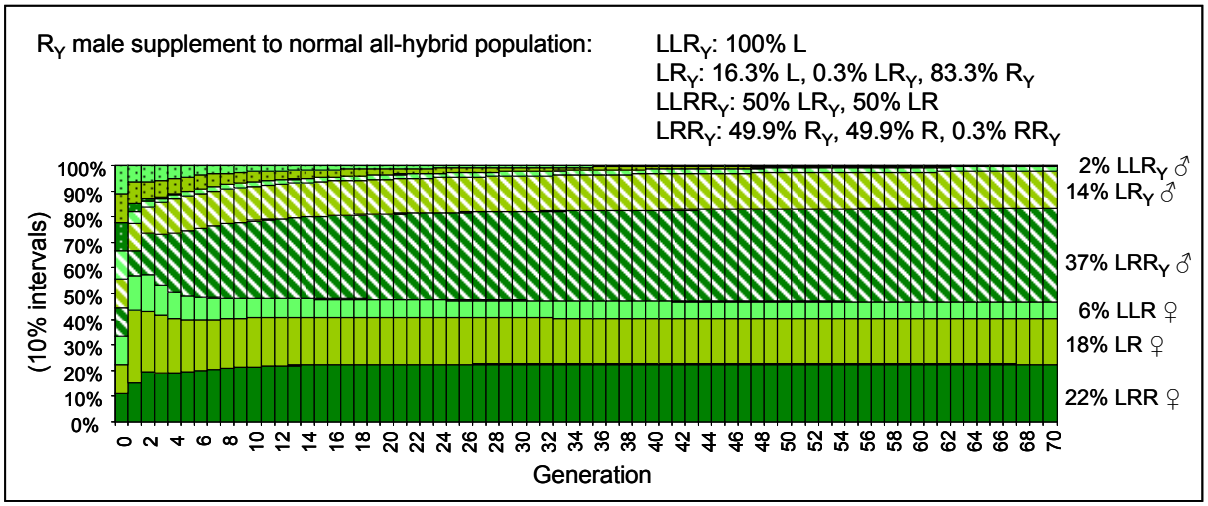

Fig 6. Development and equilibrium of a normal all-hybrid *P. esculentus* population with the addition of males with Y factor in the R genome (striped signature). Colour codes, gamete pattern and survival as in Fig 5.

To turn a normal all-hybrid population into a pure LLRR population, a more than twofold survival or reproductive output of both male and female LLRR relative to the di- and triploid hybrids was required (Fig 7; the corresponding figures for reproductive output were very similar and are therefore not shown). Increasing only female reproductive output had little effect on the proportion of LLRR; probably because LR sperm, not LR eggs, were the limiting factor in the normal all-hybrid populations. An increase in the proportion of LR sperm made by LR males made the proportion of LLRR go up, as already seen in the LRR-rich population (Fig 5). However, no more than 20% LLRR could be attained by increasing the proportion of LR gametes made by both LR males and females to 100% (data not shown). LLRR take-over was also possible by various combinations of sub-threshold values for survival, reproductive output and LR gamete production by diploids.

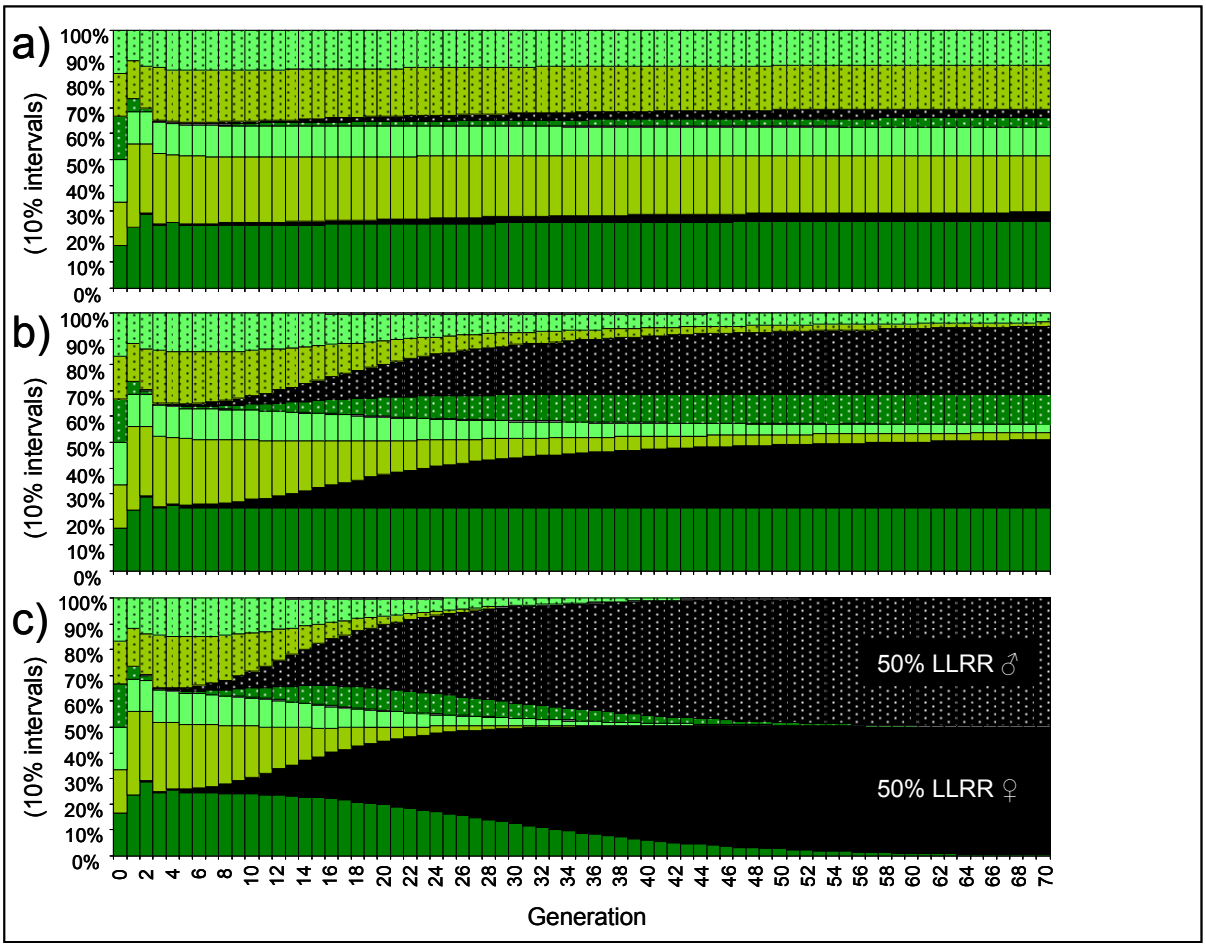

Fig 7. Development and equilibrium of a normal all-hybrid *P. esculentus* population with increased survival of LLRR tetraploids (black). LLRR survivals of a) 1.5 b) 2.0 and c) 2.3. Survival of di- and triploid hybrids = 1; non-hybrid survival = 0. Gamete pattern and colour codes as in Fig 5.

LL survival values above 0 and up to slightly beyond 0.7 lead to stable coexistence of LL and hybrids with the proportion of LL depending on their survival (Fig 8a+b; gamete

pattern for normal all-hybrid populations). With an LL survival of one, like the hybrids, the LL frogs drove the hybrids extinct (Fig 8c). Stable coexistence of RR and the usual hybrid genotypes was possible with RR survivals of up to approximately 0. 5 (Fig 8d+e); above this level only LR males and RR females remained, in proportions depending on the RR survival (Fig 8f; with the Y factor confined to the L genome, RR males could not arise). Equal survival of all genotypes (LL, RR and hybrid survival = 1) lead to the same equilibrium as in Fig 8f.

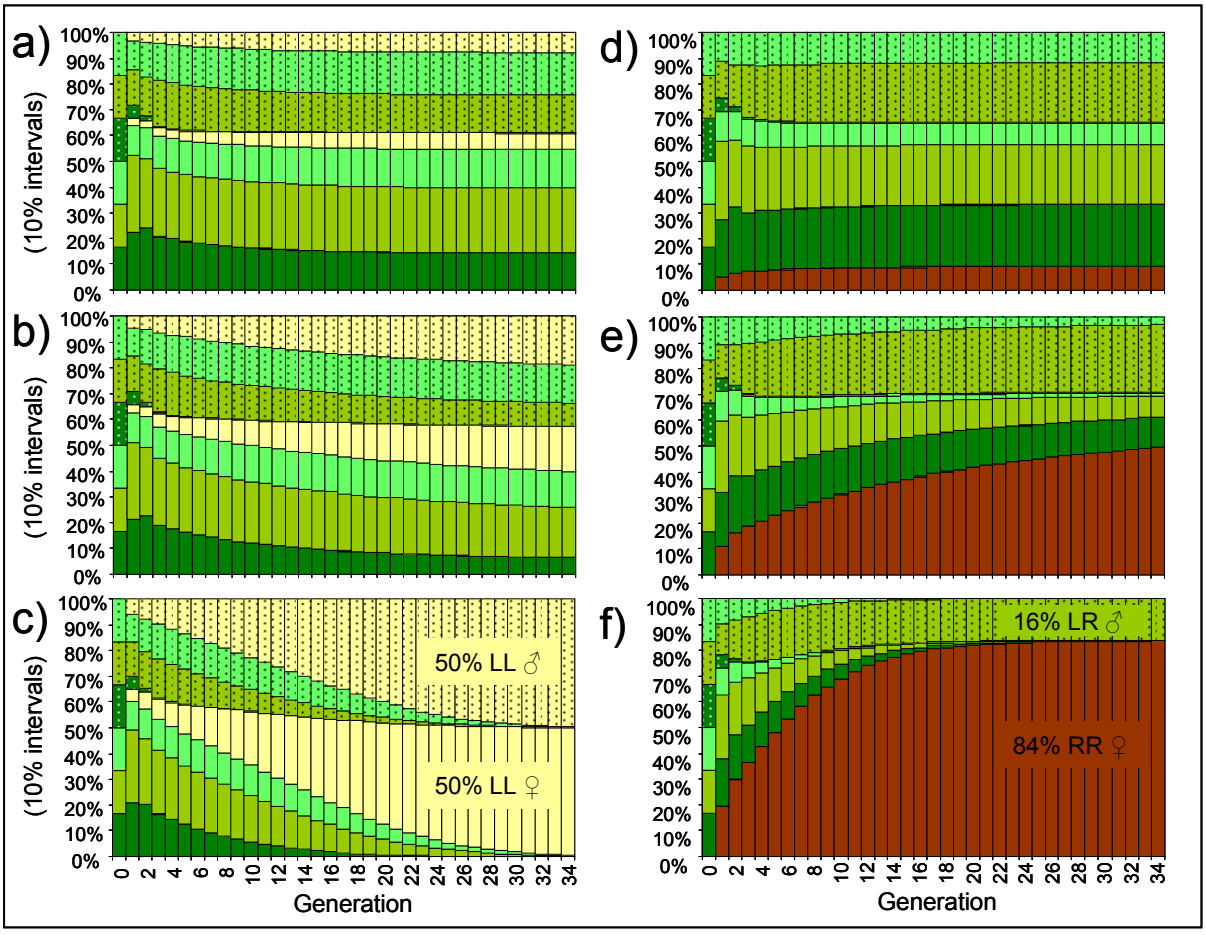

Fig 8. Development and equilibrium of a normal and initially all-hybrid *P. esculentus* population with survival of non-hybrids. LL (yellow) survivals of a) 0.5 b) 0.7 and c) 1.0. RR (brown) survivals of d) 0.2, e) 0.5 and f) 1.0. Hybrid survival = 1; survival of the other non-hybrid = 0. Colour codes and gamete pattern of the hybrids (green) as in Fig 5.

Discussion

Gamete production patterns in the crossing experiment were largely in agreement with the rough gamete pattern from earlier studies. In addition, this study quantified the proportions of LR and R eggs laid by LR females and of rare gamete types. It also confirmed that the male-determining Y factor was confined to the L genome in the populations tested. The LRR males present in some of these populations could thus not have arisen from R genomes with Y

factor. Nevertheless, the riddle of the LRR males was solved: 22% of the sperm from LR males from LRR-rich populations was LR sperm which, according to the model, was sufficient to explain the high proportion of LRR males in these proportions. In contrast, it remains unresolved whether LR females play a role in shaping genotype proportions in natural populations. Based on the gamete results, the model predicted adult equilibrium genotype compositions which are compared to empirical data below. The model also suggested that the proportion of tetraploid LLRR remains low unless their survival and/or reproductive output is substantially increased, and that inviability of non-hybrid genotypes is a precondition for the maintenance of the all-hybrid populations.

Gamete types

As expected from other studies, triploid frogs made almost exclusively haploid gametes with the genome they had in double dose. The only exception was LLR females that produced 11.1-11.5% LL gametes. Similar results were obtained with Swedish frogs by Jakob and Arioli [26]. They had an overall mean of 4.75% LL eggs from LLR females (n = 4), while their remaining triploids produced almost no unusual gamete types. Rare LL and RR ova are known from several areas and population types [32-35]. Reports on frequent LL gametes from Hungary [36], France [11] and possibly the Czech Republic [21] concern sperm – not eggs as in the present study. The Hungarian LL sperm was unreduced (made by apomixis) as opposed to most or all of the LL eggs, the RR egg and the RR sperm in the present study (made by automixis). Thus, LL gametes can apparently be formed by different cytological processes. Automixis also occurs in LR frogs: after the L genome is excluded, the R genome is not directly passed on into gametes, but first undergoes duplication and meiosis [37].

With 81.7% diploid LR and 18.1% haploid R eggs, R egg production in diploid females was also within the range of previous estimates (21.4%, n = 7, [24]; 25.0%, n = 4, [25]; 8.8%, n = 10, [26]). Together with the 18.1% (n = 19) in the present study, the overall, weighed average becomes 17.1% based on 40 frogs. Unfortunately for future studies, a count of large and small eggs gave an unreliable estimate of the ploidy in the viable offspring, because the small eggs often had very mixed genotypes and/or high mortality.

Sex determination

Sex determination in vertebrates can be either environmental or genetic, but only genetic sex determination has been found in amphibian populations studied [38, 39]. Both XY and WZ systems exist and shifts between them have been extraordinarily frequent in amphibian

evolution [38]; XY and WZ systems can even coexist within the same species [40]. Amphibian sex chromosomes show little or no differentiation [38], which might explain the viability of polyploid amphibians: with no Y chromosome degeneration there is no X dosage compensation to be disrupted by polyploidy, as opposed to in for example birds and mammals [41]. However, the lack of sex chromosome differentiation complicates the study of sex determination, so that markers for DNA-sexing have been obtained for very few species [42].

In the present study, which was the first to investigate sex determination in the Scandinavian all-hybrid populations, sex was therefore determined by dissection. This gave slightly inconsistent results: 19-20 male and 2-3 female offspring had the wrong sex compared to the expectations from an L genome-confined Y factor. Low frequencies of the unexpected sex were also obtained in other studies and for unknown reasons ([43] and references therein). The reasons are unknown, but one possible explanation could be underdeveloped gonads, which are small and round and look like small testes (own observation in Swiss F1 hybrids). *P. esculentus* is known to have retarded ovary development, apparently because the special hybrid mode of gametogenesis creates complications [44]. Alternatively, unexpected offspring sex could result from spontaneous mixed genotypes. A missing Y factor could render unexpected females and a substitution or addition of a Y factor to an otherwise pure R sperm could render unexpected males.

LRR males

LRR males arose from LR sperm fertilizing R eggs in the crossing experiment, which suggests that LR sperm is responsible for their existence in some natural ponds. The 22% LR sperm from LR males found in the crossing experiment was sufficient to increase the LRR proportion to comprise almost half of the males and more than half of the females in the model population. The 22% LR sperm was mainly provided by only two out of seven LR males from LRR-rich populations; both from pond 089. Also in pond 089, Jakob and Arioli [26] found LR sperm in only one of three LR males, resulting in an overall mean of 4% LR sperm among them. In Alsønderup – the other LRR-rich population investigated – one out of four LR males produced LR sperm, i.e. none in the present, but one in a previous study [24]. With such large individual differences, large samples are required for reliable estimates.

As explained in the introduction, LRR males could also originate from R genomes with a Y factor and/or from RR eggs. R genomes with a Y factor are unlikely to occur, since in a sample of 15 LR and LRR males from LRR-rich populations no R genome with a Y factor was found, although the model predicted that, if present, they should spread and

eventually replace L genomes with Y factors. It can still not be ruled out that RR eggs contribute to the formation of LRR males, as only four LRR females from LRR-rich ponds were investigated. Elevated proportions of RR eggs were, however, not observed in these four LRR females [26].

Gamete patterns might drive adult genotype proportions

LR sperm was apparently more common in LRR-rich populations than in normal populations in the present study, and for L sperm the trend was opposite. Similar striking differences in the sperm types between LR frogs from LLR-rich, LR-rich and LRR-rich populations were observed by Jacob and Arioli [26]. In contrast to in the present study, LR females in the study of Jacob and Arioli [26] also made more R eggs in LR-rich populations than in triploid-rich populations. Although in both studies, the sample sizes are too small for a statistic confirmation of these apparent population type-specific differences, the data suggests that gamete patterns may drive the genotype proportion in all-hybrid populations of *P. esculentus*. As extensive efforts to show relations between adult genotype proportions and ecological factors has been of rather limited success [26] this suggestion is a welcome alternative hypothesis that needs proper testing. If true, the number of evolutionary significant units relevant for conservation might be higher in *P. esculentus* than presently realized.

Modelled versus natural populations

The genotype proportions predicted for normal and LRR-rich all-hybrid populations matched available field data from a large sample of natural Swedish ponds and a subsample of LRR-rich ponds, respectively. The large sample consisted of 3000 frogs from 12-23 Swedish ponds with various genotype compositions sampled over 3 years [26]. Within males, the among-year range (compared to the model result in parentheses) was 33-60 (43) % LLR, 36-60 (57) % LR, 2-4 (0) % LRR; within females there were 15-28 (19) % LLR, 26-44 (42) % LR and 39-45 (39) % LRR. In the LRR-rich pond 089 there were within males (n = 103) 17 (14) % LLR, 58 (23) % LR, 5 (20) % LLRR, 20 (43) % LRR; within females (n = 216) 6 (9) % LLR, 29 (25) % LR, 0 (6) LLRR and 65 (59) % LRR [26]. The fit with Alsønderup was less good, but here the sample size was only 46 frogs.

The overall good fit between observed and modelled genotype proportions suggests that the model captured the essence of al least the normal all-hybrid populations, in spite of its simplicity. The simplifications included random mating, equal survival of LLR, LR and LRR and equal reproductive output for all genotypes, and were mainly motivated by insufficient

empirical data. In the LE system, females prefer LL to LR males [45-47], but it is not known if females can and do distinguish between male genotypes in the all-hybrid populations. Concerning survival, LR probably survived better than triploids from eggs to 1-year-olds [25], but thereafter differences between genotypes disappeared. A capture-mark-recapture study on the Swedish ponds showed that local adult survival differed between sexes and genotypes, but overall the genotypes had similar annual survivals of around 30% (n = 329 [26]). In contrast, poor survival of LRR has been suggested by authors based in other areas [33, 48-50]. With respect to reproductive output, female fecundity depends on both female body size and genotype [26, 51], while fertilization success is apparently reduced in LR males producing several kinds of sperm [18, 26, 52]. In addition, reproductive output also depends on the genotype-specific proportion of aneuploid eggs and sperm that do not give rise to viable offspring. Such data is lacking; the present study only suggested that most eggs that died or were aneuploid came from LR females. Furthermore, male mating success is also important for the reproductive output of males. In the LE system, LL males with scramble competition behaviour have more mating success than territorial LR males [53], but no data are available on genotype-specific mating success of LLR, LR and LRR males.

Evolutionary potential of all-hybrid populations

According to the model, LLRR frogs needed a more than twofold advantage in either reproductive output, survival or a combination, to turn a normal or LRR-rich population into a pure LLRR population. In vertebrates, polyploidy tends to have little or no effect on body size [54], so no increased fecundity in females is expected. Increased reproductive output in LLRR is not unlikely because tetraploidy may result in more regular meiotic processes and, hence, a higher proportion of fertile gametes for both sexes. As this advantage should arise spontaneously in LLRR, and it has not yet helped LLRR increase in frequency, it is, however, unlikely to make LLRR increase further in the future. Concerning survival, field data from the Swedish study area do not suggest that LLRR have a selective advantage over the other genotypes. On the contrary: the proportion of LLRR decreased from 2.8% at the egg stage to zero at metamorphosis and among one-year old juveniles [25]. In ponds 089 and Alsønderup, the proportion of LLRR adults was also lower than expected from the gametogenetic pattern (see above). Although a broad variety of habitats have been investigated, it is, however, possible that the LLRR would have higher survival in a different habitat.

A recent study of the hybridogenetic Iberian minnow, *Squalius alburnoides*, provides strong evidence that di- and triploid hybrid populations can be an intermediate step on the

way to a tetraploid species [9]. *S. alburnoides* (also called *Leuciscus, Rutilus* and *Tropidophoxinellus)* resembles *P. esculentus* most of the five other hybridogenetic complexes presently known. Most populations of this freshwater fish are composed of diploid and triploid hybrids, one parental species and sometimes backcrossed males of the other, now extinct, parental species [55]. Symmetrical tetraploids are common in low proportions, though not as low as in *P. esculentus*. In contrast, two newly discovered populations have 73% tetraploids with even sex ratios, normal meiosis and the capability to reproduce among themselves [9]. In addition, postzygotic isolation appears to have arisen between triploid and tetraploid forms. It was suggested that the success of the tetraploids is connected to the more upstream habitat of these populations, but this needs further investigation. These discoveries suggest that tetraploidization could also happen in *P. esculentus* if it be given sufficient habitat variation, space and time to evolve. Maybe it has even happened somewhere already and can be found if looked for.

Threats to all-hybrid populations

Normal all-hybrid populations will, according to the model, only persist when survival of LL and RR is zero. With moderate survival of parental genotypes, stable mixed populations of hybrids and non-hybrids will result; with LL survival above approximately 0.7 or RR survival above approximately 0.5, all or most hybrid genotypes go extinct. These results call attention to the possibility that introduction of water frogs is a potential threat to all-hybrid populations. It is not known why LL and RR genotypes have lower survival in natural all-hybrid populations. One possible explanation is that the parental species are at a selective disadvantage under Scandinavian environmental conditions. This explanation is, however, not very plausible, since *P. ridibundus* does occur on the very nearby Danish island of Bornholm, and *P. lessonae* lives both north, south and east of the all-hybrid populations. Another, not mutually exclusive, explanation is that the parental genotypes are homozygous for deleterious alleles that have become almost or entirely fixed in the genetically generally very depleted L and R genomes [25, 27].

If the reduced fitness of non-hybrids is due to homozygosity for deleterious mutations, an interesting question is what would happen if *P. lessonae*, *P. ridibundus* or *P. esculentus* with fewer or different deleterious mutations were introduced into the all-hybrid populations. Would they give rise to viable non-hybrids, spread and radically change the system; maybe even drive (most of) the hybrid genotypes extinct? Such a situation is currently observed in the Swiss LE system, where RR offspring used to die due to homozygosity of deleterious

mutations [13], but now in many places survive and take over due to introductions of *P. ridibundus* [56]. Or would the viable alleles be scattered and swamped out by recombination in non-hybrids and triploids with the more numerous resident genomes? This is possibly the situation in northern Germany, where all-hybrid populations apparently persist without geographic isolation from *P. lessonae* and *P. ridibundus* populations further south. The answer might depend on the number of loci with deleterious alleles in high frequencies and on the number of introduced genomes without deleterious mutations at these loci. If introduced frogs include *P. ridibundus* males, however, the risk seems high that their R_y will be invasive and replace L_y in the recipient all-hybrid populations, with the previously mentioned consequences of modified equilibrium genotype proportions and slightly increased hybrid load.

Conclusions

In the model, the gamete data from the crossings produced an equilibrium distribution for normal, i.e. average and common, all-hybrid populations that matched empirical data from a large sample of Swedish frogs and ponds. Furthermore, the 22% LR sperm produced by LR males from LRR-rich populations could explain the high proportions of the normally very rare LRR males in these uncommon LRR-rich populations. These results thus fill major gaps in our understanding of this unusual and fascinating breeding system. Furthermore they strongly suggest that differences in genotype proportions between ponds are gamete-pattern-driven, although further studies with larger sample sizes are required to confirm this. The consequences of gamete-pattern-driven population differentiation would be large. All-hybrid populations now appear to constitute not one, but several intrinsically different breeding systems with different dynamics and fitness. The realization of this diversity makes the system more fascinating, more important to conserve, but also more difficult to study, as generalizations are less applicable.

Tetraploidization is, according to the model, unlikely to happen by a change of gamete patterns alone, but requires a more than twofold increase in survival or reproductive output of both male and female LLRR. As exemplified by the Iberian minnow, *S. alburnoides*, an increase in tetraploid fitness might be achieved in a different habitat.

R genomes with Y factor would, according to the model, be invasive and change the all-hybrid populations, if *P. ridibundus* males were introduced. The model also predicted that survival of LL or RR genotypes would lead them to invade and possibly even replace the hybrid populations. Without knowing the causes of non-hybrid inviability in the all-hybrid

populations, it can, however, not be predicted if introduction of foreign *P. lessonae*, *P. ridibundus* or *P. esculentus* would increase the survival of LL and RR and thus be a threat to the all-hybrid populations.

P. esculentus appears to be a large natural experiment where several breeding systems and many population types develop and are tested in our time. Speciation may take place while we can watch and learn from the process. Geographic isolation is, however, often an important factor in speciation [57]. Let us therefore hope that our increasing rate of translocating plants and animals will not ruin this potential opportunity for water frogs to speciate and the opportunity for us to study it.

Acknowledgements

My warmest thanks to Lars Iversen and Ursina Tobler for help with the field part of the crossing experiment and to Sandra Rötlisberger for the laboratory part of the DNA analysis. My gratitude also goes to Heinz-Ulrich Reyer for financial and moral support and helpful comments on the manuscript. Thanks to Homayoun Bagheri for advice on the design of the model result figures. This study was funded by the Swiss National Science Foundation (grant no. 31-64004.00 to H.-U. Reyer). Finally, I am grateful to Daniel Leutwyler for marrying me in spite of having to help killing metamorphs on one of our first dates.

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Additional file 1.
Inferring gamete types from allele data

Allele genome specificity

Genome specificity was inferred by applying the full set of 18 primer pairs to morphologically identified *P. lessonae* and *P. ridibundus* from Poland, the Danish island of Bornholm, Estonia and Latvia, respectively (Christiansen 2005 and own unpublished data). The genome specificity of new alleles in the Swedish and Danish *P. esculentus* could be inferred from the ones with known specificity. The thus inferred genome specificity of the alleles that occurred in the 269 Swedish and Danish adult frogs analyzed for the present study is shown in Table A.

Genomic composition of frogs and gametes

The genomic composition (LLL, LL, LLLR, LLR, LR, LLRR, LRR, LRRR, RR RRR or "mixed") of adults and offspring was determined from allele tables like Table B; it shows the "full cross" 16N, where tadpoles were raised to metamorphosis and analyzed with all 18 primer pairs.

Genomic composition was primarily inferred from the four primer pairs showing dosage effect, i.e. differences in the relative height of the L and R peaks amplified (Christiansen 2005). The method cannot distinguish LLL from LL, LR from LLRR or RR from RRR without heterozygosity at other loci, but with 18 primers in total, heterozygosity should have sufficed for detecting most cases of LLL, LLRR and RRR. For the loci without dosage effect, the genomic composition was assumed to agree with the dosage effect loci. Assumed duplicate alleles and assumed absence of alleles (–) were put in parenthesis.

After deducing the full genotypes of all parents and offspring, the parental contribution to each offspring could be inferred. In Table B, four loci (blue alleles) indicate that all 13 offspring obtained their R genome from their father, while one (offspring 6) got an additional paternal L genome. Two loci (red alleles) indicate that all offspring got one or two L genomes from their mother. From this cross it can be deduced that the male made 12 R sperm, 1 LR sperm and that the female made 6 L eggs and 7 LL eggs. Mean gamete proportions were calculated from all the crosses parented by each frog.

Table A. Genome specificity for the alleles found in 269 Swedish and Danish *P. esculentus* adults caught

Primer	CA1b6 (dosage)		Res16 (dosage)		RICA1b5 (dosage)		Ga1a19rdsgn (dosage)		Re1CAGA10	
Genome	L	R	L	R	L	R	L	R	L	R
Alleles found	78	85, 92, 96	121	123, 127	119	132, 134	195	199, 201, 205	90, 96	95, 95, 108, 110, 125, 127

Background colour indicates genome specificity (yellow = L genome, orange = R genome).

Table B. Genotype table for adults and offspring in cross 16N

Individual	Sex	Cross	Geno	CA1b6 (dosage)				Res16 (dosage)				RICA1b5 (dosage)				Ga1a19rdsgn (dosage)				Re1CAGA10			
Male M26		16	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	96	(-)	108	(-)
Female F5		16	LLR	78	78	85	-	121	121	127	-	119	119	134	-	195	195	201	-	96	(96)	108	(-)
Offspring 1	f	16N	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	?	(-)	108	(-)
Offspring 2	f	16N	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	?	(-)	108	(-)
Offspring 3	f	16N	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	?	(-)	108	(-)
Offspring 4	f	16N	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	96	(-)	108	(-)
Offspring 5	m	16N	LR	78	-	85	-	121	-	127	-	119	-	134	-	195	-	201	-	96	(-)	108	(-)
Offspring 6	m	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	?	(?)	108	(-)
Offspring 7	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 8	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 9	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 10	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 11	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 12	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)
Offspring 13	f	16N	LLR	78	78	85	85	121	121	127	127	119	119	134	134	195	195	201	201	96	(96)	108	(-)

Background colour indicates genome specificity (yellow = L genome, orange = R genome).
Blue alleles are from the male parent; red alleles from the female parent.

Table A continued

Primer	Rrid059Ardsgn		RICA2a34		Rrid013A		Res20	RICA5	ReGA1a23	RICA1a27
Genome	L	R	R	L	R	L	L	L	L	L
Alleles found	278	309, 314, 322	106	134, 145, 147, 150, 150	281, 287	296, 299	121,123	156, 160	117, 119, 123, 125, 127	95, 112, 116, 120,

Table B continued

Individual	Sex	Cross	Geno	Rrid059Ardsgn				RICA2a34				Rrid013A				Res20		RICA5		ReGA1a23		RICA1a27	
Male M26		16	LR	278	(-)	314	(-)	106	(-)	147	(-)	281	(-)	299	(-)	121	(-)	260	(-)	123	(-)	116	(-)
Female F5		16	LLR	278	(278)	309	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	260	123	(123)	95	116
Offspring 1	f	16N	LR	278	(-)	314	(-)	106	(-)	145	(-)	281	(-)	296	(-)	121	(-)	260	(-)	123	(-)	95	(-)
Offspring 2	f	16N	LR	278	(-)	314	(-)	106	(-)	145	(-)	281	(-)	296	(-)	121	(-)	260	(-)	123	(-)	95	(-)
Offspring 3	f	16N	LR	278	(-)	314	(-)	106	(-)	145	(-)	281	(-)	296	(-)	121	(-)	260	(-)	123	(-)	95	(-)
Offspring 4	f	16N	LR	278	(-)	314	(-)	106	(-)	145	(-)	281	(-)	296	(-)	121	(-)	260	(-)	123	(-)	116	(-)
Offspring 5	m	16N	LR	278	(-)	314	(-)	106	(-)	145	(-)	281	(-)	296	(-)	121	(-)	256	(-)	123	(-)	116	(-)
Offspring 6	m	16N	LLR	278	(278)	314	(-)	106	(-)	145	147	281	(-)	296	299	121	(121)	260	(260)	123	(123)	116	(116)
Offspring 7	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	260	123	(123)	95	116
Offspring 8	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	260	(260)	123	(123)	95	116
Offspring 9	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	260	123	(123)	95	116
Offspring 10	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	260	123	(123)	95	116
Offspring 11	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	260	123	(123)	95	116
Offspring 12	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	260	(260)	123	(123)	95	116
Offspring 13	f	16N	LLR	278	(278)	314	(-)	106	(-)	145	(145)	281	(-)	296	(296)	121	(121)	256	(256)	123	(123)	95	116

Table A continued

Primer	RICA18	Re2CAGA3	Rrid064A	Res22	Rrid169A	Rrid135A
Genome	L	L	L	R	R	R
Alleles found	180, 186	169, 200, 112, 116, 220	211, 225, 227	84, 110, 114, 119	187, 189, 191, 201	168, 203

Table B continued

Individual	Sex	Cross	Geno	RICA18		Re2CAGA3		Rrid064A		Res22		Rrid169A		Rrid135A	
Male M26		16	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Female F5		16	LLR	186	(186)	212	(-)	225	(-)	84	(-)	187	(-)	203	(-)
Offspring 1	f	16N	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 2	f	16N	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 3	f	16N	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 4	f	16N	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 5	m	16N	LR	186	(-)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 6	m	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 7	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 8	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 9	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 10	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 11	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 12	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)
Offspring 13	f	16N	LLR	186	(186)	216	(-)	211	(-)	110	(-)	187	(-)	203	(-)

Heterozygosity, recombination and automixis

Within-genome heterozygosity enabled detection of recombination and automixis; this is why the most heterozygous triploids were preferred for the crossings. In Table B, the mother was heterozygous at the L loci R1CA5 and R1CAa27.

Among the six offspring in Table B that received just one L genome from their mother (offspring 1-6), three combinations of alleles were observed at R1CA5 and R1CAa27, namely 260+95, 260+116 and 256+116. This suggests that triploid frogs provide within-genome recombination (Christiansen and Reyer 2009).

Among the seven offspring that originated from LL eggs (offspring 7-13), four (7, 9, 10 and 11) were heterozygous for both RlCA5 and RlCA1a27, while in the other three (8, 12 and 13) one of these loci was homozygous and the other was heterozygous. This reduction in heterozygosity, compared to the mother, in a high proportion of LL eggs, suggests that LL eggs are made by automixis; i.e. that the two L genomes undergo duplication and meiosis or meiosis and fusion.

Egg size and mixed genotypes

Table C depicts a summary table for determining genomic compositions in cross 13C; an "LR female" cross where the focus was on egg ploidy and offspring were only reared to the beginning of the feeding stage. The size classes reflected ploidy in that large eggs resulted in triploid offspring whereas small eggs mainly resulted in offspring of lower ploidy (the exception is offspring 7, which is mainly triploid).

An additional difference between large and small eggs was, however, striking: The loci analyzed agreed on genomic composition for offspring 11-20 from large eggs (except for one locus in offspring 19), whereas they strongly disagreed for offspring 1-9 from small eggs. The latter offspring could either be interpreted as LR with various numbers of missing R alleles, or as LL with extra R alleles (it is not known if single Ls represent one or more Ls). This example illustrates what is meant by "badly mixed genotypes" in the article and it highlights the difficulties of classifying such genotypes. Since all mixed genotypes were verified by additional PCR rounds, they are unlikely to reflect methodological mistakes. Instead, they may arise either from aneuploidy (with one L genome and half an R genome from the mother) or from recombination between the L and R genomes in the mother (from eggs being a mixture of LL and LR)."Badly mixed genotypes" appear to be inviable since high frequencies were observed only among early larval stages; not among metamorphs or adults.

Table C. Summary genome composition table for adults and offspring in cross 13C.

Individual	Sex	Cross	Geno	RICA1b6	Res16	RICA1b5	Ga1a19	RICA2a34	Res20	RICA5	Re2Caga3	Rrid064A
				L R dosage	L R dosage	L R dosage	L R dosage	L R	L	L	R	R
Random male			LLR	LLR	LLR	LLR	LLR	LLR	L	LL	R	R
Female F22			LR	LR	LR	LR	LR	LR	L	L	R	R
Offspring 1	?	13C small	LRmix	LR	L	LR	LR	LLR	L	L	R	R
Offspring 2	?	13C small	LRmix	LR	L	L	LR	LR	L	L	null	R
Offspring 3	?	13C small	LRmix	LR	L	LR	LR	L	L	L	R	null
Offspring 4	?	13C small	LRmix	LR	L	LLR	LR	L	L	L	R	null
Offspring 5	?	13C small	LRmix	L	L	LR	L	L	L	L	null	null
Offspring 6	?	13C small	LRmix	LR	L	LR	LR	L	L	L	null	R
Offspring 7	?	13C small	LLRmix	LLR	LLR	LLR	LLR	LLR	L	L	R	null
Offspring 8	?	13C small	LRmix	LR	L	LR	LR	LR	L	L	null	R
Offspring 9	?	13C small	LRmix	L	LR	L	L	L	L	L	R	R
Offspring 11	?	13C large	LLR	LLR	LLR	LLR	LLR	LLR	L(L)	L(L)	R	R
Offspring 12	?	13C large	LLR	LLR	LLR	LLR	LLR	LLR	L(L)	L(L)	R	R
Offspring 13	?	13C large	LLR	LLR	LLR	LLR	LLR	LLR	L(L)	LL	R	R
Offspring 14	?	13C large	LLR	LLR	LLR	LLR	LLR	LLR	L(L)	L(L)	R	R
Offspring 15	?	13C large	LLR	LLR	LLR	LLR	LLR	L(L)R	L(L)	LL	R	R
Offspring 16	?	13C large	LLR	LLR	LLR	LLR	LLR	L(L)R	L(L)	L(L)	R	R
Offspring 17	?	13C large	LLR	LLR	LLR	LLR	LLR	LLR	L(L)	L(L)	R	R
Offspring 18	?	13C large	LLR	LLR	LLR	LLR	LLR	L(L)R	L(L)	LL	R	R
Offspring 19	?	13C large	LLRmix	LLR	L(L?)	LLR	LLR	L(L)R	L(L)	LL	R	R
Offspring 20	?	13C large	LLR	LLR	LLR	LLR	LLR	L(L)R	L(L)	L(L)	R	R

Blue alleles are from the male parent; red alleles from the female parent. Black alleles are alleles shared by the parents.

Additional file 2.

Sperm genotype proportions in *P. esculentus* males

Male parent					Sperm									
Genotype	Pop. type	Pond	Male	Cross	n	LL%	(sons)	L%	(sons)	LR%	(sons)	R%	(sons)	RR%	(sons)
LLR	LLR-rich	001	M1	5	55			100	-54.5						
LLR	LLR-rich	001	M2	6	54			100	-66.7						
LLRmix	LLR-rich	001	M3	6	58			100	(5.2)[1]						
LLR	normal	By32A	M4	2	44	0.0-2.3[a]	0	100	-58.1						
LLR	normal	108	M5	2	41			100	-70.7						
LLR	normal	108	M6	3	56			100	-55.4						
LLR	normal	Road	M7	4	30			100	-33.3						
LLR	normal	By32A	M8	4	40			100	-52.5						
LLRmix	normal	126	M9	6	40			100	-37.5						
LLR	normal	108	M10	16	46			100	-43.5						
LLR	LRR-rich	89	M11	2	35			100	-60						
LLR	LRR-rich	89	M12	3	32			100	-40.6						
LR	normal	111	M13	2	41			36.62	-100			61	-12	2.4	0
LR	normal	32A	M14	3	42			19.02	-100	4.8[b]	50)	76.2	-3.1		
LRmix	normal	By32A	M15	4	31							100	0		
LR	normal	108	M16	5	50							100	-6		
LR	normal	126	M17	5	47							100	0		
LR	normal	111	M18	6	55			58.2	-100			41.8	-4.3		
LR	normal	111	M19	16	56							100	0		
LR	LRR-rich	089	M20	3	25					56.5	-84.6	43.5	-0.2		
LR	LRR-rich	089	M21	3	43							100	0		
LR	LRR-rich	Alsø	M22	4	43							100	-7		
LR	LRR-rich	Alsø	M23	5	50							100	-2		
LR	LRR-rich	Alsø	M24	6	53							100	-1.9		
LR	LRR-rich	089	M25	16	32					93.8	-100	6.3	-50		
LR	LRR-rich	089	M26	16	39					2.6	-100	97.4	-2.6		
LRR	LRR-rich	089	M27	3	29							100	0		
LRR	LRR-rich	Alsø	M28	4	34							100	-5.9		
LRR	LRR-rich	Alsø	M29	5	53							98.1	0	1.9	0
LRR	LRR-rich	138	M30	5	52							100	-1.9		
LRR	LRR-rich	Alsø	M31	6	58							100	-1.7		
LRR	LRR-rich	089	M32	16	53							100	0		

[1] Note, almost no sons.
[2] L sperm underrepresented in first cross with L eggs.
[a] corresponds to a in Additional file 3.
[b] corresponds to b in Additional file 3.

Additional file 3.

Egg genotype proportions in *P. esculentus* females

Female parent					1st*	Large eggs*		Small eggs*		Eggs genotyped, all sipships						
Genotype	Pop. type	Pond	Female	Cross	n*	%	geno**	%	geno**	n	LL%	L%	LR%	R%	RR%	LLR%
LLR	LLR-rich	001	F1	3	374	-	-	-	-	75	0.0-1.3[b]	100				
LLR	LLR-rich	102	F2	6	261	-	-	-	-	83		100				
LLR	normal	126	F3	4	41	-	-	-	-	40	15	85				
LLR	normal	111	F4	5	428	-	-	-	-	83	6	94				
LLR	normal	108	F5	16	476	-	-	-	-	60	41.7	58.3				
LLRmix	LR-rich	By011	F6	6	371	-	-	-	-	68	14.7	85.3				
LLR	LRR-rich	089	F7	2	458	-	-	-	-	50	0.0-2.0[a]	100				
LR	LLR-rich	102	F8	3	361	5.3	LR	94.7	R	83			5.3	94.7		
LR	LLR-rich	001	F9	4	186	-	-	-	-	73			100			
LR	LLR-rich	102	F10	13	507	76.9	LR	23.1	R	20			76.9	23.1		
LR	LLR-rich	102	F11	14	389	28	LR	72	R	25			26.1	72	1.9	
LR	LLR-rich	102	F12	15	433	-	-	-	-	12			100			
LR	LLR-rich	102	F13	17	458	15.9	LR	84.1	R	23			15.9	84.1		
LR	LR-rich	By011	F14	2	271	-	-	-	-	59			100			
LR	LR-rich	By011	F15	5	413	-	-	-	-	84			98.8			1.2
LR	LR-rich	By011	F16	13	411	85.6	LR	14.4	(mix)	40			100			
LR	LR-rich	By011	F17	14	610	50	LR	50	R	20			50	50		
LR	LR-rich	By011	F18	15	530	-	-	-	-	10			100			
LR	LR-rich	By011	F19	17	472	-	-	-	-	22			100			
LR	LRR-rich	089	F20	6	276	98.2	LR	1.8	(mix)	88			100			
LR	LRR-rich	089	F21	16	333	94.3	LR	5.7	(mix)	68			100			
LR	LRR-rich	089	F22	13	513	94.3	LR	5.7	(mix)	34			100			
LRmix	LRR-rich	089	F23	14	351	94	LR	6	(mix)	20			100			
LR	LRR-rich	089	F24	15	401	96.3	LR	3.7	R +mix	20			98.1	1.9		
LR	LRR-rich	089	F25	17	501	-	-	-	-	19			100			
LRR	normal	126	F26	2	466	0.2	RR	99.8	R	61				99.8	0.2	
LRR	normal	126	F27	4	348	-	-	-	-	69				100		
LRR	normal	108	F28	5	283	13.8	(died)	86.2	R	87				98.9	1.1	
LRR	normal	032	F29	5	652	0.2	(died)	99.8	R	65				100		
LRR	normal	108	F30	6	82	4.9	(died)	95.1	R	83				100		
LRR	normal	032	F31	16	633	0.2	RR	99.8	R	64				99.8	0.2	
LRR	LR-rich	By011	F32	3	260	0.4	(RR died)	99.6	R	75				100		
LRRmix	LRR-rich	089	F33	16	234	6.4	(mix died)	93.6	R	45				100		

***** eggs in the first sipship only. Offspring from up to ten large and up to ten small eggs were genotyped.

****** prevailing egg genotype in size class.

- means that there was only one size class or that the sorting attempted did not reflect the egg content.

[a] corresponds to a in Additional file 2.

[b] corresponds to b in Additional file 2.

Additional file 4.

P. esculentus population model

Easy-to-use model that instantly yields a graph of how your *P. esculentus* population develops over time in response to initial adult genotype proportions, gamete genotypes and relative offspring survival.

Available at:
http://www.biomedcentral.com/1471-2148/9/135/additional/

Later version published in BMC Ecology 2010, vol 10, article 14

Coexistence of diploid and triploid hybrid water frogs: population differences persist in the apparent absence of differential survival

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Abstract

The role of differential selection in determining the geographic distribution of genotypes in hybrid systems has long been discussed, but not settled. The present study aimed to asses the importance of selection in structuring all-hybrid *Pelophylax esculentus* populations. These populations, in which the parental species (*P. lessonae* with genotype LL and *P. ridibundus* with genotype RR) are absent, have different pond-specific proportions of diploid (LR) and triploid (LLR and LRR) genotypes. Here, with data from 12 Swedish ponds, we first show that in spite of significant genotype proportion changes over time, the most extreme ponds retained their differences over a six years' study period. The predominance of different genotypes in different ponds could be a consequence of differential selection varying between ponds (selection hypothesis), or, alternatively, of different gamete production patterns among ponds (gamete pattern hypothesis). The selection hypothesis was tested in adults by a mark-recapture study in all 12 ponds. As the relative survival and proportion of LLR, LR and LRR did not correlate within ponds, this study provided no evidence for the selection hypothesis in adults. Then, both hypotheses were tested simultaneously in juvenile stages (eggs, tadpoles, metamorphs and one year-old froglets) in three of the ponds. A gradual approach to adult genotype proportions through successive stages would support the selection hypotheses, whereas the presence of adult genotype proportions already at the egg stage would support the gamete pattern hypothesis. The result was a weak preference for the gamete pattern hypothesis. These results thus suggest that selection is of little importance for shaping genotype distributions of all-hybrid populations of *P. esculentus*, but that further studies are

needed for a confirmation. Moreover, the study provided valuable data on genotype-specific body lengths, adult survival and sex ratios.

Introduction

Species coexistence is believed to be niche-based (Silvertown 2004). However, for hybrid complexes, opinions differ as to whether environment-specific differential selection is important for the geographic distribution and diversity of hybrid genotypes. Theory has been developed along two lines, both represented by two opposing, but not mutually exclusive models. Within each line, the first model assumes environment-specific differential selection on hybrids, whereas the alternative model assumes that selection on hybrids does not vary among environments. The first line applies to the mixture of hybrids and their parental species (the tension zone model, Barton and Hewitt 1985; and the bounded hybrid superiority model, e.g. Moore 1977). The second line concerns hybrid clones (the frozen niche variation model, Vrijenhoek 1979; and the general purpose genotype model, e.g.Lynch 1984). However, for hybrids that are neither sympatric with their parental species, nor clonal, theories have not been formulated and, consequently, the role of differential selection in determining the geographic distribution and diversity of such hybrids is unknown.

The edible frog, *Pelophylax esculentus* (called *Rana esculenta* until Frost et al 2006) constitutes an example of a hybrid that can form all-hybrid populations that are neither sympatric with parental species (Arioli 2007 chap. 5; Jakob 2007 chap. 2) nor clonal (Christiansen and Reyer 2009). These hybrids demonstrate such extreme hybrid superiority that parental species continuously arising from hybrid x hybrid matings are constantly outcompeted (Christiansen et al 2005; Arioli 2007 chap. 3) and thus virtually absent among adults. Still, various genotype classes are present, as the hybrids include both diploid and triploid forms. Genotype proportions have been observed to vary between ponds, and it remains to be assessed whether differential selection among ponds is responsible. The current study aimed to find and test relevant hypotheses with and without environmental variation in differential selection to explain the genotype distributions in these very special all-hybrid *P. esculentus* populations.

Within the genus of water frogs, *Pelophylax*, the edible frog, *P. esculentus* (genotypes LLR, LR and LRR), arose and still arises by matings between the pool frog, *P. lessonae* (genotype LL), and the marsh frog, *P. ridibundus* (genotype RR, i.e. Graf and Polls Pelaz 1989). As indicated by the names, the two parental species have different habitat preferences within their largely overlapping distribution areas that cover most of Europe. The smaller *P.*

lessonae lives in pools and ponds while the larger *P. ridibundus* prefers lakes and river-influenced water bodies (Pagano et al 2001; Plötner 2005 and references therein). The *P. esculentus* hybrids have the broader habitat tolerance and usually co-occur with at least one of the parental species.

The hybrids reproduce by hybridogenesis, which implies that genetic recombination does normally not take place between L and R genomes in hybrids. Instead, gametes contain one or the other genome, or both, but not a mixture. Hybrids are thus formed anew every generation by the fusion of two gametes with different genomic contents. In the all-hybrid populations of Southern Sweden that were investigated in this study, LLR frogs of both sexes make mostly L gametes (and LLR females make a small fraction of LL eggs), LRR of both sexes make R gametes, LR females make LR and some R eggs while LR males make R and rarer LR or L sperm (Figure 1, Arioli 2007 chap. 1; Jakob 2007 chap. 5; Christiansen 2009). When two L or two R gametes combine, offspring with parental species genotypes (LL and RR) arise, but they die before sexual maturity under natural conditions (Christiansen et al 2005; Arioli 2007 chap. 3). Sex determination is an XX-XY system with a male-determining Y factor located in one L genome in males (Christiansen 2009). As a consequence, LRR males are rare, except in ponds with high frequencies of LR sperm (Christiansen 2009). Tetraploids are also rare (Jakob 2007 chap. 2). The remaining five hybrid genotypes, LLR and LR males, LLR, LR and LR females, are frequent in almost all ponds (Jakob 2007 chap. 2).

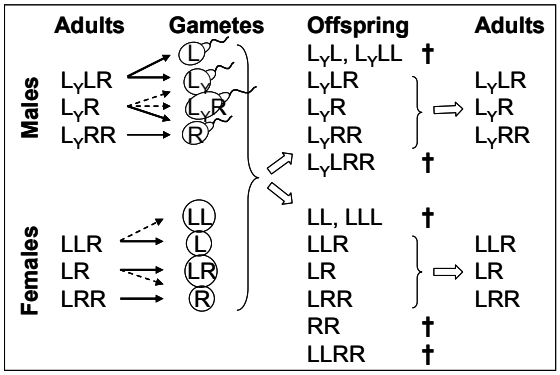

Figure 1. Gamete production and population maintenance of all-hybrid *P. esculentus* populations.

Because the various genotypes propagate each other rather than themselves (cf. Figure 1), the populations are self-sustaining and should constantly be drawn to a gamete pattern-determined stable equilibrium (Som and Reyer 2006a; Christiansen 2009). Yet, variation in the proportions of LLR, LR and LRR between ponds has been observed (Christiansen et al 2005; Jakob 2007 chap. 2). It remains to be established how such variation can be generated; especially if it does not result from stochastic events, but is maintained over time. Two

hypotheses have been proposed to explain such persistent equilibrium differences: 1) Variation in selection regimes between ponds; here called the selection hypothesis (Jakob 2007 chap. 3). 2) Variation in gamete patterns between ponds; here called the gamete pattern hypothesis (Christiansen 2009). A third possibility is that both selection and gamete pattern contribute to the variation in genotype compositions between ponds. They could either act antagonistically or pond-specific gamete patterns could be adapted to the local selection regime.

The selection hypothesis is based on the observation of differences between *P. lessonae*, *P. ridibundus* and *P. esculentus* in adult habitat preference and in larval performance under various ecological conditions, as found by a variety of studies (Pagano et al 2001; Peter et al 2002; Anholt et al 2005). Ecological differences could thus also exist within hybrid genotypes, i.e. between LLR, LR and LRR. Such differences could either be a consequence of a dosage effect, as observed in morphometry (callus size divided by tibia length), where there is a cline from LL through LLR, LR and LRR to RR (Fog et al 1997 p. 238; Pagano and Joly 1999; Christiansen 2005; Jakob 2007 chap. 1), or as a consequence of triploids having larger cells, as observed in erythrocytes (Schmeller et al 2001; Jakob 2007 chap. 1). The only study comparing fitness among LLR, LR and LRR in different environments was, however, little conclusive: The prevalence of adult LLR was positively correlated with human constructions and that of LR adults with forest around the breeding pond, but the majority of ecological parameters measured were not significantly correlated with genotype proportions (Jakob 2007 chap. 3).

The gamete pattern hypothesis is based on a study showing a tendency for variation in gamete patterns between ponds (Christiansen 2009). The sample size was not sufficiently large to document significant differences, given the large variation between individuals, but the trend pointed in a direction that could explain the difference in genotype proportions between so-called “normal” and “LRR-rich” populations.

Distinguishing between the selection and gamete pattern hypotheses does not only help us understand how these intriguing all-hybrid populations function; it has consequences for our perception of this and other breeding systems. The selection hypothesis would suggest that the all-hybrid populations of *P. esculentus* constitute one breeding system with different appearances under different ecological conditions. The gamete pattern hypothesis would suggest that the all-hybrid populations are a mosaic of intrinsically different variants of this breeding system. The latter would, in other words, suggest a breeding system with high biodiversity and various evolutionary significant units.

In this study, we first document the adult genotype frequencies over six years in a sample of 12 Swedish ponds to investigate whether different temporally stable population types exist. It is also determined whether body length increases with R/L dosage effect from LLR through LR to LRR, or whether body length is larger in adult triploids (LLR and LRR) than diploids (LR).

Secondly, we test the selection hypothesis in adults by investigating LLR-, LR- and LRR-specific survival rates in each of the 12 ponds. Survival rates were estimated from mark-recapture data, and the effect of genotype, sex, time and season on survival was determined by model selection. Since genotype (LLR, LR, LRR) is not heritable (cf. Figure 1), survival is the only relevant measure of fitness in this system. If selection at the adult stage is responsible for a pond being dominated by one genotype, then the proportion and survival of each genotype in the 12 ponds should be positively correlated.

Thirdly, we test the selection hypothesis and the gamete pattern hypothesis simultaneously at juvenile stages in a subset of three ponds, i.e. the most extreme LLR-rich, LR-rich and LRR-rich ponds. Genotype proportions were assessed in samples of eggs, tadpoles, metamorphs and one year-old froglets from the three ponds. If selection is responsible for one genotype being dominant among adults in a certain pond, this genotype is expected to rise in frequency during juvenile stages. Alternatively, if the gamete pattern is responsible for the adult genotype frequency, the genotype that is dominant among adults is expected to be dominant already in the egg stage.

Methods

Adult sampling

The study was performed in 12 ponds in Skåne (Scania), Southern Sweden (Figure 2, coordinates in Jakob 2007 chap. 2). In each of the 12 ponds, a sample of adult frogs was caught twice per year, with catching dates differing between ponds and years (Appendix A). The frogs were caught at night by hand or dip net, dazzling them with a torch and moving about with waders and sometimes a small rubber boat. Especially in later years, an effort was made to obtain at least thirty adults per catching round, including at least ten individuals of each sex. To obtain these numbers, catching rounds could extend over several days (or, rarer, weeks); the catching date was then calculated as the mean date on which the frogs were caught. Due to removal of floating vegetation, the number of frogs in pond 014 was so low in 2006 and 2007 that additional sampling in the 10m distant neighbouring pond was necessary for reaching reasonable sample sizes.

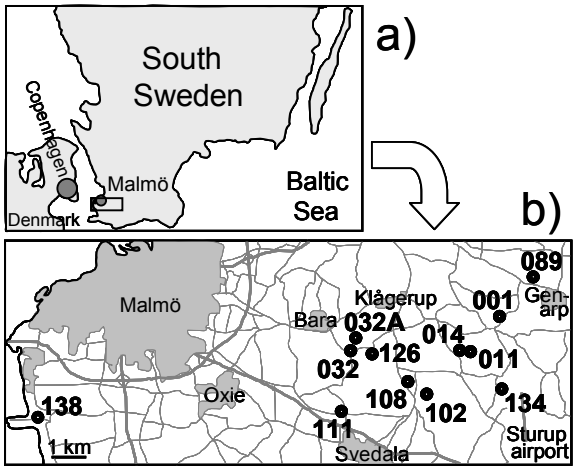

Figure 2. Geographic locations of a) the study area in Southern Sweden (rectangle) and b) the 12 ponds within the study area.

The frogs were brought to the nearby Stensoffa Field Station, Torna Hällestad, for processing. New frogs were measured from snout to vent (spine straight) with a slide calliper, were individually marked with a PIT tag transponder (Trovan ID101, Euro I.D., DE) and had a toe-tip cut off for microsatellite genotyping. In 2002-2004 also a blood sample was taken for genotyping by DNA flow cytometry. Recaptured frogs were just measured and identified via the PIT tag. All frogs were returned to their source pond within a few days of sampling, with the exception of minor numbers used in crossing experiments in 2002, 2004 and 2006.
The present study considered adult frogs only, which were defined as individuals with at least 55 mm from snout to vent.

Offspring sampling

Ponds 001, 011 and 089 had the most divergent genotype distributions, and were therefore picked for the study of juvenile stages. In 2006, these three ponds were sampled for eggs, tadpoles and metamorphs and in 2007 for one year-olds as judged by their size.

Eggs were sampled June 5 to 30 in which period the ponds were searched for new egg clutches every three days. From every clutch judged by location, egg sizes and age to be from a different female than the neighbouring clutches, a small subsample of approximately 20 eggs was brought to the field station. Upon reaching the free-swimming feeding stage, one healthy-looking tadpole per clutch was randomly chosen for rearing to metamorphosis, whereas the remaining tadpoles were returned to the pond they were collected from. Healthy-looking tadpoles were preferred for rearing because abnormal tadpoles were assumed to die under natural conditions and thus not provide information on population maintenance. The chosen tadpoles were reared in outdoors 40-liter tubs with up to 15 tadpoles per tub and food

ad libitum (as described in Christiansen and Reyer 2009). In total, tadpoles were reared from 44, 61 and 65 egg clutches from ponds 001, 011 and 089, respectively, and DNA data were obtained from 95% of them, i.e. from 40, 60, and 62 individuals, respectively. Sex was determined by dissection approximately a week after tail resorption (as described in Christiansen 2009). For statistic analysis, the eggs were divided into two equal-sized groups according to sampling date: sample 1 constituted the earlier collected eggs and sample 2 the later collected eggs.

Tadpoles were sampled by dip-netting; 30 on July 6 (sample 1) and 30 on July 20 (sample 2) in each of the three ponds. On July 20, the tadpoles showed large size variation, but intermediate-sized tadpoles were preferred. The tadpoles were reared and analyzed as described for the eggs. Of the 60 tadpoles sampled in each pond, DNA samples were obtained from 58, 59, and 59 (97%), respectively.

Metamorphs were sampled on August 1, 6, 11, 16, 21 and 31. On each of these dates, 10 metamorphs with a few millimetres of unresorbed tail were sampled in each of the three ponds. The metamorphs were all sexed and DNA-analyzed after ten days of rearing. For statistic analysis, the metamorphs from the first three dates were pooled and labelled sample 1 whereas those from the last three dates constituted sample 2.

One year-olds were sampled in 2007. Per pond, 30 were sampled in May (sample 1) and 30 in July (sample 2). As one year-olds were not nearly as numerous as offspring at the earlier stages, they were not sacrificed and sexed, but were released to their source pond after removal of one or two toes for DNA analysis.

Genotyping

The adult frogs caught 2002-2004 were genotyped using a combination of microsatellite analysis and DNA flow cytometry as described in Jakob (2007 chap. 1). The adults and offspring from 2005-2007 were genotyped using microsatellite analysis of four loci with dosage effect, capable of distinguishing LL, LLR, LR and LRR and RR (Christiansen 2005; Christiansen and Reyer 2009). General agreement between the two genotyping protocols was confirmed in frogs analyzed with both protocols because they were caught in both time periods.

Some frogs had mixed genotypes where microsatellite loci and/or flow cytometry analyses disagreed on the genotype. As the resolution for identifying mixed genotypes varied with the methods applied, no analyses on mixed genotypes were possible. The 45 frogs with mixed genotypes from 2005-2007 were assigned to LLR, LR or LRR according to the

majority of the loci analyzed (sometimes more than the four dosage effect loci were analyzed). Likewise, the 23 frogs from 2002-2004 recorded to have mixed diploid genotypes were included in the data set as LR. However, 27 frogs from 2002-2004 recorded to have mixed or mosaic triploid genotypes were excluded from the data set, as it was unsure whether they had most resemblance with LLR or LRR.

Analysis of adult genotype proportions and lengths

First, it was tested whether genotype proportions differed systematically between the first and the second catching round per year. If not, the two catching rounds per year could be pooled to increase sample sizes, or used as replicates.

Ternary plots were used for displaying the proportion data for three genotypes (LLR, LR and LRR) in two dimensions. The ternary plots were drawn in the programme Past, version 1.80 (Hammer et al 2001). Ternary confidence areas were drawn with programmes provided by Gert Jan Weltje (Weltje 2002) and the software Grapher (version 7, Golden Software Inc, Golden, Colorado, USA).

In the ternary plots and the following statistical analyses of adult genotype proportions, males and females were treated separately, because our sampling did not necessarily reflect the natural sex ratio. Sometimes our sampling might have been biased by the different behaviour of the two sexes; at other times we biased the sample sex ratio ourselves in order to obtain at least ten individuals of the rarer sex.

For investigating whether different temporally stable population types exist, the effects of pond and year on genotype proportions were analyzed. Year was treated as a discrete variable as continuous temporal changes could not be expected, because they would imply the ultimate extinction of one or more genotypes. The data were analyzed with generalized linear models (GLMs) in the programme R version 2.8.0 (R Development Core Team 2008). GLMs with binomial error distributions (logit) have the advantage of coping with proportion data in a way where sample sizes are taken into account. Pooling the two annual catching rounds to avoid very small samples was therefore not necessary. Using a binomial error distribution requires binomial data, as for example “LLR male” and “non-LRR male”. Therefore, a model was fitted separately to each genotype within sex (mLLR, mLR, mLRR within males and fLLR, fLR, fLRR within females). For most models, the data exhibited overdispersion (residual deviation > the degrees of freedom), wherefore quasibinomial error distributions and F tests were used. Fitting a model to all three genotypes (LLR, LR and LRR) within each sex is statistically redundant, because the genotype proportions add up to one and the result for

the last genotype is therefore given by the first two analyses. Nevertheless, to facilitate reading and interpretation, tests for all three genotypes are provided in the results tables for this and subsequent analyses. However, when Bonferroni-correcting the significance level, α, the apparently three tests for LLR, LR and LRR only count as two; thus tests for mLLR, mLR, mLRR, fLLR, fLR and fLRR count as four tests. Strict Bonferroni corrections were used, because sequential Bonferroni corrections would differ according to which two tests are considered redundant.

Differences in adult body length as a function of sex, genotype, pond and their interactions were analyzed with an ANOVA in R (R Development Core Team 2008).

Testing the selection hypothesis in adults

The selection hypothesis implies that the proportion of each genotype in a pond is affected by its relative survival. For testing the hypotheses in adults, survival probabilities therefore had to be estimated from the six year's mark-recapture data and correlated with the genotype proportion data presented above.

Adult survival rates were estimated using the Comack-Jolly-Seber mark-recapture model. The ponds had to be analyzed separately, because their catching dates, and thus time intervals between catching rounds, differed. These time intervals were expressed in fractions of years so that the output survival estimates were in units of years.

The Comack-Jolly-Seber model assumes that all marked individuals within predefined groups (here mLLR, mLR, mLRR, fLLR, fLR and fLRR) have the same probability of recapture and of survival, that marks are not lost and that sampling events are short compared to the time intervals between samplings (Cooch and White 7th edition). First, the goodness of fit of the data to the assumptions was tested in U-care vers. 2.02 (Pradel et al 2003; Choquet et al 2005). Included in this programme are tests for transience (animals migrating through the population) and trap-dependence (animals liking or avoiding capture).

Models were constructed and selected in the programme MARK (Cooch and White 7th edition). MARK estimates Φ = survival rate and p = recapture probability – each as a function of parameters of interests. A set of candidate models are evaluated by AICc according to how well they minimize deviance from the data with the fewest parameters possible (Cooch and White 7th edition). In the present study, the full model was Φ(genotype*sex*time) p(genotype*sex*time), where genotype was LLR, LR or LRR, sex was male or female and time was time-dependence; i.e. varying survival over the 11 time intervals between the 12 catching rounds. The remaining candidate models were reduced versions of this full model.

Some candidate models included season, as a reduced alternative to time. The season parameter implied difference in survival in the summer period between within-year catching rounds as opposed to the period between catching round two in one year and catching round one the following year. A model assuming constant parameters, symbolized by a dot, was also included among the candidate models for p and Φ.

The analysis was done in three steps. First, the best models for recapture probability were selected for every pond. This was done by keeping Φ constant while evaluating different models for p. All models with ΔAICc below two, and thus with the best fits, were selected for the next steps of the analysis. Secondly, the best model(s) for p were inserted into the candidate models estimating survival (Φ), and these survival models were evaluated using AICc. Finally, mean yearly survival rates over the six years were calculated by averaging over all the survival models that did not contain time or season, weighed by the AICc weight by each model.

Testing for the selection hypothesis among adults implied testing for correlation between the proportion (within sex) and relative survival within each genotype in R (R Development Core Team 2008). Spearman rank correlations were used because proportion data have non-normally distributed residuals and were furthermore highly overdispersed in a GLM with genotype proportion as dependent variable and relative survival as independent variable. Relative survival rates for mLLR, mLR, (mLRR,) fLLR, fLR, and fLRR were calculated as their difference from the pond mean. These relative survival rates were preferred to absolute survival rates because pond differences in absolute survival could otherwise blur the correlation.

Testing both hypotheses in juvenile stages

The analysis of juvenile stages was made to investigate how pond differences in adult genotype composition arise, i.e. why pond 001 have more LLR, pond 011 more LR and pond 089 more LRR when compared to each other. The selection hypothesis predicts that the differences arise by differential selection, so that for example in pond 001, the proportion of LLR increases more during subsequent juvenile stages than in the other ponds. Thus, a significantly higher slope for LLR in pond one as compared to ponds 011 and 089 would support the selection hypothesis, and similarly for the slopes of LR in pond 011 and LRR in pond 089. In contrast, the gamete pattern hypothesis predicts that the differences in adult genotype compositions between ponds are a result of differential gamete production by the same genotypes in different ponds. In pond 001 a high proportion of LLR should thus be

present already in the egg stage of new generations. Thus a significantly higher intercept of LLR in pond 001 than in ponds 011 and 089 would support the gamete pattern hypothesis, and similarly for the intercepts of LR in pond 011 and of LRR in pond 089.

To test for such differences in slopes and intercepts, three GLMs were fitted; one for LLR, one for LR and one for LRR (though the last was redundant). In the LLR model, pond 001 was first (used as intercept), in the LR model pond 011 was first, and in the LRR model pond 089 was first. Males and females were pooled within genotypes, as their proportions were not expected to be biased by behaviour and sampling technique, and sex data were not available for the one year-olds. As for adults, quasibinomial error distributions were used. Stage (eggs, tadpoles, metamorphs, one year-olds) was coded as a continuous variable (0, 1, 2, 3) with eggs as stage zero so that the fitted value at this stage would be the intercept. Pond was treated as a discrete variable.

Results

Adult genotype proportions

Including recaptures, we caught 5051 LLR, LR and LRR frogs above 55mm in the 12 Swedish ponds during two annual catching rounds, 2002 to 2007 (listed in Appendix A). Among males, there were 42.6% LLR, 53.2% LR and 4.2% LRR; among females, 18.2% LLR, 45.0% LR and 36.9% LRR. The sex ratio in the sample was 40.4% males and 59.6% females, but this might be biased by differential behaviour and sampling effort (see methods). In addition, a total of six LL frogs were caught; no RR frogs were encountered. Four different individuals (caught a total of seven times) were classified as LLRR by DNA flow cytometry. Excluding the six LL and the seven LLRR captures plus 27 captures of triploids with uncertain genotypes, the mean sample size per catching round per pond was 14.2 (range 2-43) for males and 20.9 (range 1-71) for females.

No overall season effects were found between catching rounds one and two as tested over all ponds and years (paired t-tests: mLLR: $t_{71} = 1.865$, P = 0.066; mLR: $t_{71} = -1.746$, P = 0.085, mLRR: $t_{71} = 0.067$, P = 0.947; fLLR $t_{71} = -0.742$, P = 0.472; fLR $t_{71} = 0.151$, P = 0.880; fLRR $t_{71} = 0.635$, P = 0.528; Bonferroni-corrected $\alpha_4 = 0.0125$). This analysis might not reveal season effects differing among ponds or years, but a detailed visual inspection of increases and decreases between catching rounds one and two revealed no patterns. In the following analyses, the first and second catching rounds were therefore pooled or used as replicates.

The genotype proportions obtained in the 12 Swedish ponds over the six years are illustrated in Figure 3a. The two annual catching rounds were pooled to increase the sample sizes, which thus became mean 28.3 (range 6-64) for males and mean 41.8 (range 9-97) for females. By providing examples of 95% confidence intervals for similar sample sizes, Figure 3b suggests that most of the year-to-year variation observed within ponds is due to sampling stochasticity. From inspection of Figure 3a it is evident that ponds 001, 011 and 089 were the most different ponds with non-overlapping genotype distributions for either sex.

The GLM analyses of genotype frequency on year and pond (both categorical) showed a very highly significant effect of pond for all genotypes (Table 1). The effect of year was smaller, but nevertheless highly significant for most genotypes, especially LR and LRR females. In addition, interaction of pond and year was significant for half of the genotypes.

The mean difference in genotype composition between catching rounds, as measured by their distance in a ternary plot, was smallest between catching rounds from the same year (Figure 4). For males, the differences peaked or stabilized after three years, whereas for females the genotype differences continued to increase with time. As the pairwise means are not independent, the trends of Figure 4 cannot be tested by correlation or regression. Instead, a Mantel test with permutation would be needed. However, Mantel test programmes do usually not take missing values, and the present data set had many missing values, as between-pond comparisons would have been meaningless.

The year effect in LR and LRR females (Table 1) and the increasing female genotype composition differences over time (Figure 4) both reflect the fact that the proportion of LR females increased during the study period at the expense of LRR females (Figure 5). Also inspection of Figure 3 reveals that only pond 032 did not show a net increase in LR females between 2002 and 2007.

In conclusion, at least some of the ponds were consistently different over time, although some temporal - though not seasonal - change in genotype composition was also observed. The largest temporal change was an increase in LR females that occurred in most ponds in parallel, thus not diminishing pond differences.

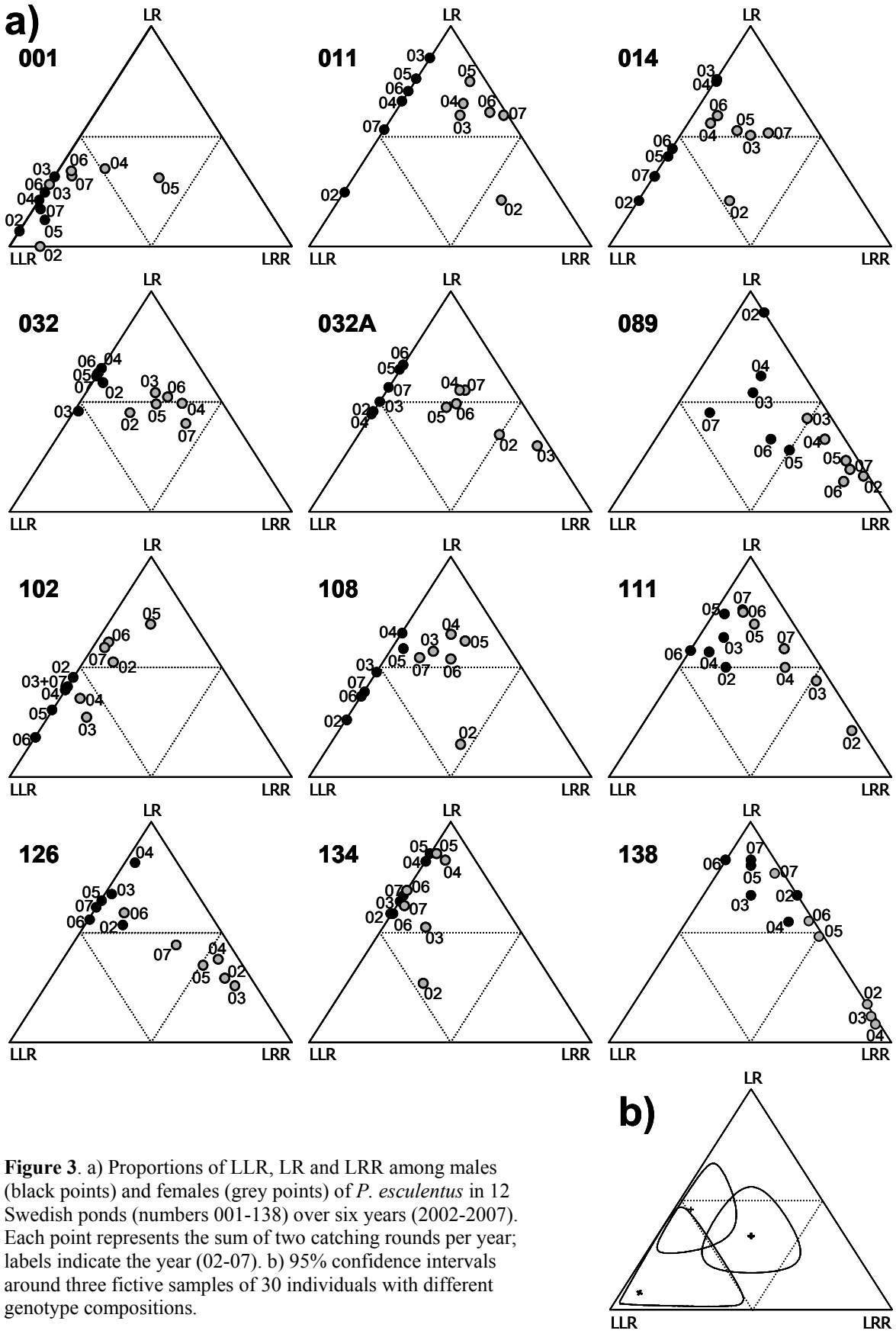

Figure 3. a) Proportions of LLR, LR and LRR among males (black points) and females (grey points) of *P. esculentus* in 12 Swedish ponds (numbers 001-138) over six years (2002-2007). Each point represents the sum of two catching rounds per year; labels indicate the year (02-07). b) 95% confidence intervals around three fictive samples of 30 individuals with different genotype compositions.

Table 1. GLM with year and pond as categorical variables (n=144 catches, df = 72)

	Res. dev.	Year (df = 5) F	P	Pond (df = 11) F	P	Interaction (df = 55) F	P
mLLR	90.87	5.57	0.0002***	20.41	< 2.20e-16 ***	1.56	0.039
mLR	93.46	4.94	0.0006**	14.63	2.37e-14***	1.62	0.028
mLRR	29.72	1.82	0.120	64.40	< 2.20e-16***	2.74	3.34e-05 ***
fLLR	124.66	1.80	0.124	20.39	< 2.00e-16***	1.20	0.235
fLR	89.65	14.90	5.08e-10***	13.28	2.19e-13***	2.89	1.38e-05***
fLRR	82.81	18.38	1.02e-11***	46.49	< 2.20e-16***	4.54	1.92e-09***

* Stars indicate 0.05*, 0.01** and 0.001*** significance levels after Bonferroni-correction: $\alpha_4 = 0.0125$*, 0.0025** and 0.0003***

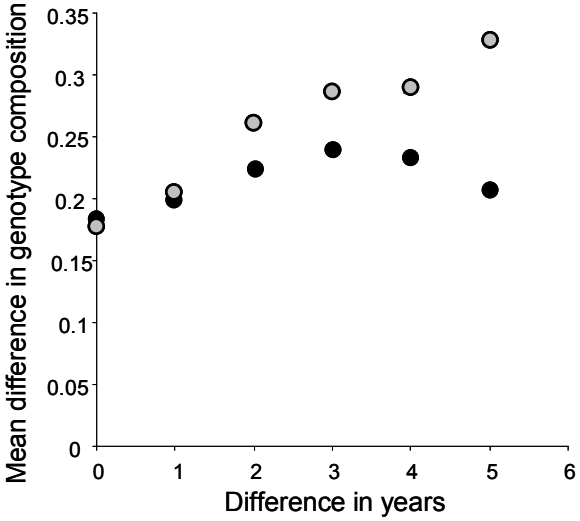

Figure 4. Mean pairwise difference in genotype composition between catching rounds within ponds as a function of the difference in years between these catching rounds. The pairwise differences in genotype composition between catching rounds were measured as their distance in a ternary plot. Black points = males, grey points = females.

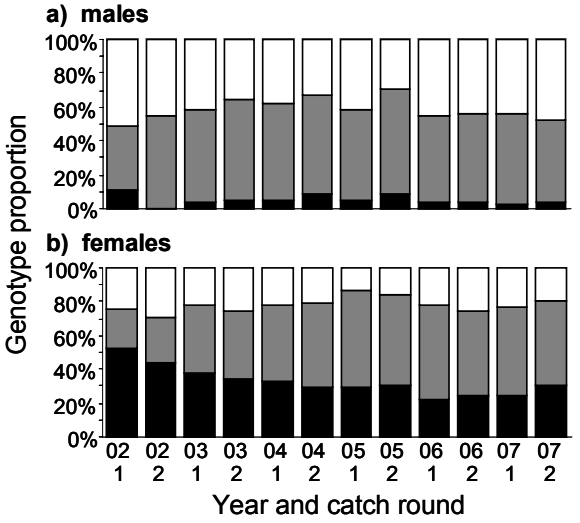

Figure 5. Temporal development of genotype proportions in a) males and b) females, summed over the 12 Swedish ponds sampled twice per year from 2002 to 2007. White = LLR, grey = LR and black = LRR.

Adult body lengths

Adult body length was measured in 4968 frogs and differed highly significantly between sexes, genotypes, ponds and all their interactions (ANOVA, F_{4900} ranged from 54.92 to

2060.44, P < 2.2e-16, for the three main effects, and F_{4900} ranged from 2.08 to 5. 54, P from 0.0056 to 5.73e-13, for the interactions). Mean length increased in the order mLLR, mLR, mLRR, fLLR, fLR, fLRR with means ±S.D. of 64.5 ±4.6, 66.0 ±5.7, 69.1 ±5.7, 72.3 ±7.1, 74.4 ±9.0 and 75.4 ±9.7 mm, respectively. Thus, males were smaller than females, as is usual in anurans, and clearly LLR<LR<LRR within sexes, indicating genome dosage effect. The means cover large variation within sexes and genotypes, as the frogs grow throughout their life.

Adult survival and the selection hypothesis

Of the 5051 adult LLR, LR and LRR frogs caught over the six years, 1011 (20.0%) were recaptures. Ninety (1.8%) of the captured individuals (mostly males) were killed by us for crossing experiments or accidentally. These were coded accordingly in the MARK input file, so that their death did not affect the survival estimates. A total of 2.8% of the previously toe-cut, recaptured frogs had lost their transponder. Only in ponds 089, 111 and 138 were LRR males sufficiently frequent to include recaptured individuals; from the remaining ponds, LRR males had to be excluded from the data set.

Goodness of fit tests in U-care showed no overdispersion. In fact, all ponds were underdispersed ($\hat{c}$ = 0.21-0.46). Only pond 001 was significant for transience (P = 0.017); no ponds were significant for trap-dependence.

Twelve combinations of the parameters genotype, sex, time and season (nested within time) were used to model both recapture probability (p) and survival (Φ). For recapture probability, eight ponds had just one best model whereas four ponds had two good models with similar fits (ΔAICc > 2). All ponds had a clear distinction in ΔAICc between the best one or two models and the poorer models. Overall, time was the most important parameter for recapture probability, as six of the 12 ponds had time as their best model (Appendix B). This reflects that sample sizes often differed over time. Season, genotype and/or sex were of highest importance in the remaining six ponds, indicating that although these factors might not have high general importance, they sometimes had high local importance. Combinations of time and other factors were not favoured. This was not surprising, since time was highly parameterized, and AIC model selection favours models that fit the data well with few parameters.

For survival, the combination of genotype, sex and time gave the best fit in eight of the 12 ponds (Appendix C). For two ponds (032 and 102) this highly parametized model did not converge and, in stead, the constant model was favoured. For only two ponds (014 and 134)

the model with genotype, sex and time was clearly rejected; in stead, models with season and constant survival, respectively, gave the best fits.

The ponds varied with respect to which genotypes and sex had the higher survival. Adult yearly survival estimates averaged 0.31 over all 12 Swedish ponds, ranging from 0.17 in pond 134 to 0.46 in pond 001 (Table 2). This average translated into a mean life span of less than 11 months as adults (lifespan = 1/ −ln(survival), Cooch and White 7th edition).

With ponds as replicates, there were no significant correlations between genotype proportion within sex and estimated relative (within pond) survival rates (Spearman rank correlation tests: mLLR: $rho_{12} = 0.245$, P = 0.437; mLR: $rho_{12} = 0.508$, P = 0.920; fLLR: $rho_{12} = 0.664$, P = 0.021; fLR: $rho_{12} = 0.252$, P = 0.424; fLRR: $rho_{12} = -0.203$, P = 0.528; Bonferroni-corrected $\alpha_5 = 0.010$; LRR males could not be tested as survival data were obtained from three ponds only). The study did thus not provide evidence for the survival hypothesis predicting that difference in survival between genotypes produces the differences in adult genotype composition observed among ponds.

Table 2. Yearly survival estimated for the six genotypes in 12 ponds.

Pond	mLLR	mLR	mLRR	fLLR	fLR	fLRR	Genotype mean
001	0.489	0.478		0.458	0.450	0.443	0.464
011	0.170	0.312		0.196	0.329	0.249	0.251
014	0.439	0.451		0.454	0.470	0.449	0.453
032	0.311	0.334		0.302	0.320	0.329	0.319
032A	0.192	0.261		0.208	0.265	0.215	0.228
089	0.440	0.437	0.444	0.365	0.368	0.369	0.404
102	0.265	0.272		0.286	0.298	0.272	0.278
108	0.293	0.165		0.398	0.224	0.077	0.232
111	0.272	0.265	0.281	0.217	0.263	0.261	0.260
126	0.281	0.268		0.297	0.279	0.308	0.287
134	0.148	0.189		0.175	0.158	0.176	0.169
138	0.347	0.624	0.564	0.272	0.578	0.047	0.406
Pond mean	0.304	0.338	0.430	0.302	0.334	0.266	0.312[1]

[1] Mean of pond means; not of genotype means.

Offspring genotype proportions and both hypotheses

The genotype distribution of the offspring sampled is shown in Figure 6. A GLM with quasibinomial error distribution was fitted for each genotype, LLR, LR and LRR (res. dev. = 81, 73 and 20 for LLR, LR and LRR respectively, with 27 df). Two of the three GLMs were significant for pond (LLR: $t_{27} = 8.51$, P = 0.003*; LR: $t_{27} = 3.65$, P = 0.047; LRR: $t_{27} = 10.03$, P = 0.001*; Bonferroni-corrected $\alpha_2 = 0.025$), but there was no significance for neither stage nor the interaction between pond and stage (data not shown).

Inspection of the regression parameters showed that in the LLR model, the intercept for pond 001 was almost significantly higher than that for pond 011 (t_{18} = -2.390, P = 0.028, Bonferroni-corrected α_2 = 0.025), and in the LRR model, the intercept for pond 089 was significantly higher than that for pond 001 (t_{18} = -2.582, P = 0.019; Bonferroni-corrected α_2 = 0.025). The remaining four intercepts and all six slopes did not differ significantly within genotypes between ponds (data not shown). As positive differences in slopes would support the selection hypothesis and positive differences in intercepts would support the gamete pattern hypothesis, this analysis thus provided no support for the selection hypothesis and only very weak support for the gamete pattern hypothesis. The offspring study had relative low discriminative power because the genotype proportions in one year-olds only incompletely matched the mean genotype proportions of adults.

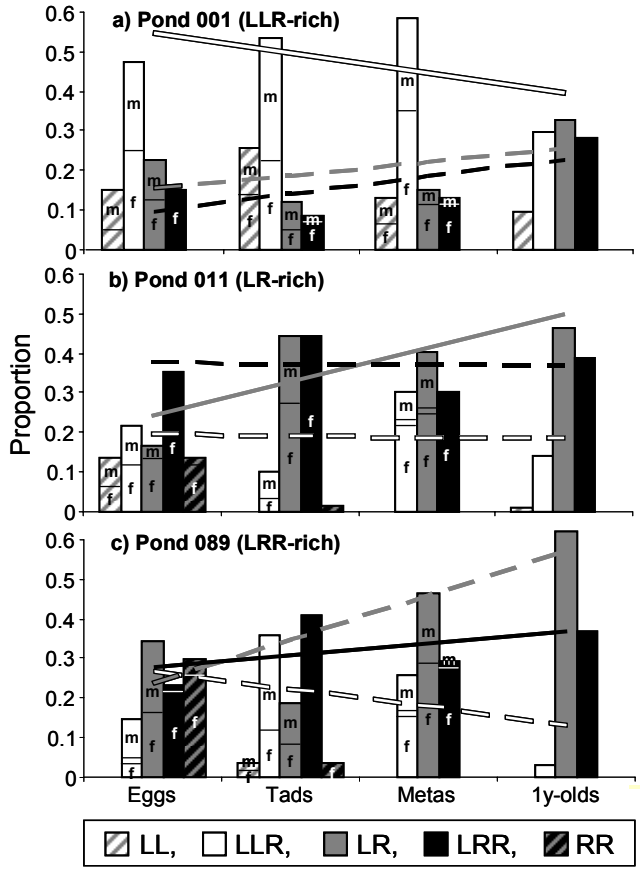

Figure 6. Genotype distributions in ponds (a) 001, (b) 011 and (c) 089 for four stages (eggs, tadpoles, metamorphs and one year-olds) of the 2006 cohort. m = males, f = females, unlabelled sections = unknown sex, i.e. the sex of one year-olds was not determined. Regression lines for LLR, LR and LRR are added for comparison of slopes and intercepts. The line is drawn solid for the genotype that is dominant among adults in the particular pond; the lines for the remaining genotypes are dashed.

Discussion

The present study is the first to thoroughly investigate the temporal stability of all-hybrid populations consisting of LLR, LR and LRR frogs. Although most genotypes exhibited temporal genotype proportion fluctuations (calculated within sex), the ponds with extreme adult genotype proportions retained their differences over the six-year study period. These

genotype composition differences could not be attributed to pond-specific selection regimes (selection hypothesis), neither in adults nor offspring. The alternative gamete pattern hypothesis was not investigated in adults in the present study, whereas the offspring study provided only very weak support for it. In the following, we will first discuss the data on body size, sex ratio and survival obtained in the present study and estimate how many generations the study period spanned. Then we will discuss problems of distinguishing between the selection and gamete pattern hypotheses, identify potential advantages and disadvantages of differential selection for the all-hybrid populations and, finally, shortly review the water frog literature on this topic.

Body size, sex ratio, survival and generation time

As adult body size constitutes an important phenotypic difference between *P. lessonae* and *P. ridibundus* with ecological implications, knowing the relative body sizes of LLR, LR and LRR can be of importance for predicting their fitness in different habitats. In the present study, a dosage effect pattern was observed among adults so that LLR < LR < LRR within both sexes, in line with *P. lessonae* being smaller and *P. ridibundus* being larger than *P. esculentus* (e.g. Plötner 2005). Thus, although triploid frogs have larger cells (Schmeller et al 2001; Jakob 2007 chap. 1), adult triploid LLR frogs were not larger than diploid LR frogs. This is in line with the general observation that in vertebrates polyploidy does not imply increased body size (Otto and Whitton 2000). Thus, although most triploids start life larger than diploids because they usually derive from larger, diploid eggs (Berger and Günther 1988; Christiansen et al 2005), this initial difference disappears. Dosage effect also applies to other phenotypic features, such as the metatarsal tubercle (callus internus) size and tibia length (Fog et al 1997; Pagano and Joly 1999; Christiansen 2005; Jakob 2007 chap. 1). The callus internus is important for digging in the partially terrestrial *P. lessonae*, while leg length is more important in the more aquatic *P. ridibundus* (Fog et al 1997 p. 255). In phenotypic and ecologic contexts, LLR and LRR are thus more different from each other than from LR, so that summarizing them as “triploids” is little informative in these contexts.

The sex ratio in *P. esculentus* is of interest because the two sexes are not expected to be produced in equal numbers (cf Figure 1). However, empirical data on sex ratio are difficult to obtain, because the observed sex ratio in field samples could have been influenced by the differential distribution of the two sexes within ponds at different times at the year and males being easier to catch from mid May to late June when they call. Thus, samples were thought to be consistently male-biased in a study of Hotz et al. (2008). In contrast, the present attempt

to catch at least 10 of each sex should have led to overestimation of the rarer sex, which was males. The overall 59.6% females found in the present study among adults, is thus an underestimate. Offspring sex ratios should be less biased, as sex-specific behaviour is not expected in eggs, tadpoles and metamorphs and the sexes could not be distinguished during catching (c.f. Vorburger 2001). Furthermore, offspring sex ratios should closer reflect initial sex ratio. The 68.1% females encountered among offspring with hybrid genotypes and sexed in the present study may thus represent the best estimate of sex ratio in this system so far. The figure fairly well matches modelling of all-hybrid populations based on gamete production by the various genotypes predicting 65.0% females (in both "normal" and LRR-rich populations, Christiansen 2009). Also in the much more widespread L-E system (*lessonae-esculentus* system), *P. esculentus* have an expected (Som and Reyer 2006b) and observed (61%, Berger et al 1988) female-biased sex ratio. In the LE system, *P. esculentus* is usually only diploid LR and always make clonal R gametes. As a consequence, the hybrids are dependent on mating with a *P. lessonae* to produce new hybrids. In an organism like *P. esculentus* where few males are needed to satisfy the mating requirements of many females, female bias has the advantage of reducing the two-fold cost of sex experienced by normal sexual species.

The mean survival of 0.31 per year found for *P. esculentus* in this study appears rather low compared to 0.61 (2 ponds, 5 and 7 years, respectively, Anholt et al 2003) and 0.53-0.70 (4 ponds, 4 years, Peter 2001) for Swiss L-E system *P. esculentus*. Both studies also analyzed mark-recapture studies with the MARK programme, but the latter also modelled migration, increasing the survival estimates. Survivals around 0.3 have, however, also been reported from tree frogs in southern Germany and Switzerland (Friedl and Klump 1997; Broquet et al 2009).

For interpreting the power of the present study on temporal stability, it is relevant how many generations the six year study period spanned. In Scandinavia, males are sexually mature when two to three years old, whereas females usually need three years to mature (Fog et al 1997). A rough average would be 2.75 years. The mean adult life span was here estimated to 11 months = 0.92 years, so the midpoint of the reproductive period should be around 2.75 + 0.5*0.92 = 3.2 years. This might be a low estimate of generation time, as female fecundity increases with body length, i.e. with age. The six years study period thus probably covered between one and a half and two generations. Even longer studies would be needed to investigate long-term development of genotype proportions in all-hybrid populations. However, the general instability of pond habitats may interfere with questions about long-term stability of frog populations.

Differential selection

The lack of evidence for the selection hypothesis in the adult survival study can be interpreted in at least two ways. Either very small survival differences are sufficient for producing the genotype proportions observed, and thus a larger number of ponds with extreme genotype proportions would be necessary for obtaining a significant correlation. Alternatively, pond variation in differential selection is not important - at least not at the adult stage. The offspring study was better suited for detecting pond variation in differential selection, because the selection potential is much larger at early stages with more individuals. Moreover, the offspring study had the advantage of testing both hypotheses simultaneously. The weak outcome was most probably due to methodological difficulties in obtaining representative samples of eggs and subsequent life stages, in spite of the effort to obtain a broad sample of especially the short-lasting egg and metamorph stages.

The only attempt this far to explain Swedish all-hybrid pond variation in genotype frequencies in terms of ecological parameters (selection hypothesis) also had limited success (Jakob 2007 chap. 3). One reason might be that the environmental axes responsible for niche differentiation cannot be determined *a priori*, (Silvertown 2004) and are not easy to identify in a wide hybrid zone without steep and obvious environmental clines. Although evidence of the gamete pattern hypothesis is this far statistically insignificant because of large individual differences between frogs of the same genotypes in the same ponds (Jakob 2007 chap. 5; Christiansen 2009), gamete analyses with larger sample sizes seems at this point the most promising approach to explaining between-pond differences in population structure. This conclusion is weakly supported by the present study.

The reproductive dependence of all genotypes in the all-hybrid populations (LLR, LR and LRR) upon each other means that differential selection is not required for coexistence of the three genotypes. It does, however, also not exclude that differential selection promote differences in genotype ratios between ponds. Differential selection could potentially have both advantages and disadvantages for the all-hybrid populations. One advantage is that niche-based coexistence can confer increased carrying capacity (Hector et al 2002; Mateos and Vrijenhoek 2002; Fridley 2003). On the other hand, differential selection could imply the disadvantage of increased hybrid load in all-hybrid populations with extreme environments, because extreme population compositions biased toward one genotype should increase the production of lethal non-hybrid genotypes. Whether all-hybrid populations can adapt to these selection pressures is also questionable, as it would require group selection. Based on these

considerations, it can not be predicted whether differential selection should occur in all-hybrid populations of *P. esculentus*.

In other water frog breeding systems, various studies have tested one or more of the four hybrid models presented in the introduction. These studies were done in the L-E (*lessonae-esculentus*) system, and one study (Pagano et al 2001) also included the very similar *perezi-grafi* system. This latter study found significant habitat differentiation among the three water frog species (*P. lessonae*, *P. ridibundus*, *P. perezi*) and their two hybrids (*P. esculentus* and *P. grafi*) in support of a mosaic hybrid zone with bounded hybrid superiority, i.e. in support of differential selection. Three studies aiming to investigate the relative importance of the frozen niche variation and the general purpose genotype models all concluded that both models may apply (Tejedo et al 2000; Hotz et al 2008; Pagano et al 2008). Finally, one study provided evidence for frozen niche variation by showing that clone mixtures of tadpoles had higher survival than monocultures (Semlitsch et al 1997). Thus all studies suggest an importance of niche differentiation / differential selection in shaping the composition of hybrid populations, although the frozen niche variation model did not have better appliance than the non-niche-based general purpose model. In conclusion, the importance of differential selection in shaping genotype proportions remains an interesting, but largely unsettled, matter in all water frog breeding systems.

Authors contributions

HUR acquired the funding and supervised the project. HUR, CJ and MA designed the mark-recapture study of adults; CJ and MA caught and genotyped adults 2002-2004 whereas DGC caught and genotyped adults 2005-2007. DGC planned and conducted the offspring study. SR participated in planning the microsatellite analyses and carried out most of the laboratory work. DGC undertook the statistical analyses and wrote the article. All authors read and approved the final manuscript.

Acknowledgements

Our warmest thanks to Lars Iversen, Ursina Tobler, Eline Embrechts, Barbara Rondinelli and Jon Loman for field assistance and to Jon Loman for mediating permits and accomodation. Josh Van Buskirk, Christoph Vorburger and Benedikt Schmidt were very kind to advice on the statistical analyses; the latter also helped improve the manuscript. The study was supported by the Swiss National Science Foundation (grant no. 31-64004.00 to H.-U. Reyer). Permits for catching, toe-clipping and marking frogs were obtained from the Swedish

authorities (Länsstyreslen I Skåne Län 522-18591-02, 522-9286-03, 522-6571-04, 522-10481-05 and Djurskyddsmyndigheten M62-05).

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Appendix A.

Number of *P. esculentus* of various genotypes (LLR, LR and LRR) caught in different ponds and years. m = male, f = female, n = total, catch = catching round.

Pond	Year	Catch	Date	Day	mLLR	mLR	mLRR	mn	fLLR	fLR	fLRR	fn	n
001	2002	1	14.05.02	14	15	1	0	16	2	0	1	3	19
001	2002	2	07.07.02	68	11	1	0	12	6	0	0	6	18
001	2003	1	22.05.03	22	9	4	0	13	5	1	0	6	19
001	2003	2	09.07.03	70	6	3	0	9	10	5	0	15	24
001	2004	1	16-18.05.04	16	25	6	0	31	6	6	3	15	46
001	2004	2	23.07.04	84	8	3	0	11	9	5	2	16	27
001	2005	1	16-22.05.05	21	15	2	0	17	2	4	8	14	31
001	2005	2	07-08.08.05	100	11	2	2	15	8	6	4	18	33
001	2006	1	14-28.05.06	22	20	9	0	29	9	9	1	19	48
001	2006	2	11.07.06	72	16	3	0	19	16	5	1	22	41
001	2007	1	25.05.07	25	21	3	0	24	8	8	0	16	40
001	2007	2	11-13.07.07	73	12	4	1	17	13	3	2	18	35
011	2002	1	22.05.02	22	6	2	0	8	1	4	7	12	20
011	2002	2	08.06.02	39	6	2	0	8	5	2	9	16	24
011	2003	1	20.05.03	20	2	8	0	10	4	19	7	30	40
011	2003	2	07.07.03	68	3	22	0	25	4	9	4	17	42
011	2004	1	15-19.05.04	16	7	31	0	38	2	26	9	37	75
011	2004	2	26.06.04	57	18	18	0	36	4	4	1	9	45
011	2005	1	12.05.05	12	6	17	0	23	0	11	2	13	36
011	2005	2	08.08.05	100	1	5	0	6	2	16	5	23	29
011	2006	1	12-28.05.06	22	6	13	0	19	2	16	6	24	43
011	2006	2	05-08.07.06	67	6	16	0	22	0	6	6	12	34
011	2007	1	22.05.07	22	7	12	0	19	0	17	7	24	43
011	2007	2	07-13.07.07	72	9	6	0	15	1	15	14	30	45
014	2002	1	23.05.02	23	5	3	0	8	7	4	2	13	21
014	2002	2	24.06.02	55	6	0	0	6	6	2	7	15	21
014	2003	1	21.05.03	21	2	14	0	16	2	13	8	23	39
014	2003	2	28.06.03	59	5	8	0	13	10	12	4	26	39
014	2004	1	25.05.04	25	5	16	0	21	4	12	1	17	38
014	2004	2	26.06.04	57	3	9	0	12	10	10	2	22	34
014	2005	1	13.-14.05.05	13	14	5	0	19	2	9	1	12	31
014	2005	2	08.08.05	100	3	7	0	10	7	8	5	20	30
014	2006	1	12.05.-10.06.06	39	12	9	0	21	4	12	1	17	38
014	2006	2	12.07.06	73	4	4	0	8	7	9	2	18	26
014	2007	1	21.05.07	21	16	9	0	25	3	6	3	12	37
014	2007	2	14.-16.07.07	76	3	0	0	3	1	6	4	11	14
032	2002	1	25.05.02	25	4	4	0	8	3	5	4	12	20
032	2002	2	13.07.02	74	7	13	1	21	4	4	0	8	29
032	2003	1	17.05.03	17	12	9	0	21	1	8	4	13	34
032	2003	2	16.06.03	47	22	20	1	43	7	12	5	24	67
032	2004	1	01.06.04	32	10	14	0	24	4	7	7	18	42
032	2004	2	14.07.04	75	5	14	0	19	2	13	8	23	42
032	2005	1	23.-25.05.05	24	11	21	0	32	10	14	8	32	64
032	2005	2	06.08.05	98	5	6	0	11	2	11	6	19	30
032	2006	1	13.05-05.06.06	32	5	8	0	13	4	14	6	24	37
032	2006	2	17-20.06.06	79	6	10	0	16	6	14	10	30	46
032	2007	1	29-30.05.07	30	4	11	0	15	3	9	9	21	36
032	2007	2	09.-10.07.07	70	6	5	0	11	5	9	10	24	35
032A	2002	1	13.07.02	74	3	2	0	5	5	7	11	23	28
032A	2002	2	16.07.02	77	3	3	0	6	1	7	9	17	23

032A	2003	1	17.05-04.06.03	33	3	2	0	5	1	6	16	23	28
032A	2003	2	22.06.03	53	2	3	0	5	1	8	15	24	29
032A	2004	1	31.05.04	31	4	0	0	4	3	7	3	13	17
032A	2004	2	15.07.04	76	6	8	0	14	3	10	5	18	32
032A	2005	1	25.-26.05.05	25	1	3	0	4	6	13	2	21	25
032A	2005	2	06.08.05	98	4	6	0	10	5	6	8	19	29
032A	2006	1	13.05-05.06.06	25	0	2	0	2	4	8	5	17	19
032A	2006	2	18.06.06	79	6	10	0	16	7	15	8	30	46
032A	2007	1	29.05.07	29	5	4	0	9	6	19	5	30	39
032A	2007	2	04.07.07	65	5	9	0	14	4	13	11	28	42
089	2002	1	11.-18.05.02	29	0	3	1	4	0	7	37	44	48
089	2002	2	08.07.02	69	0	6	0	6	1	2	9	12	18
089	2003	1	09.05.03	37	6	19	4	29	6	20	32	58	87
089	2003	2	13.07.03	74	5	8	8	21	2	20	14	36	57
089	2004	1	17.05.04	17	4	18	5	27	2	6	20	28	55
089	2004	2	22.06.04	53	2	6	4	12	2	12	13	27	39
089	2005	1	12.05.05	12	2	4	5	11	1	4	17	22	33
089	2005	2	03.08.05	95	2	1	4	7	1	7	17	25	32
089	2006	1	12.-28.05.06	18	6	8	11	25	5	9	44	58	83
089	2006	2	08.-09.07.06	69	7	8	9	24	4	3	23	30	54
089	2007	1	21.05.07	21	13	8	2	23	3	5	22	30	53
089	2007	2	10.-13.07.07	71	3	9	3	15	0	7	25	32	47
102	2002	1	26.06.02	57	4	4	0	8	4	6	1	11	19
102	2002	2	14.07.02	75	8	6	0	14	3	4	1	8	22
102	2003	1	19.05.03	19	5	3	0	8	3	2	0	5	13
102	2003	2	08.07.03	69	9	7	0	16	10	4	3	17	33
102	2004	1	12.05.04	12	3	2	0	5	5	1	0	6	11
102	2004	2	16.07.04	77	3	2	0	5	3	4	1	8	13
102	2005	1	18.05.05	18	13	3	0	16	2	9	1	12	28
102	2005	2	01.08.05	93	5	5	0	10	3	14	4	21	31
102	2006	1	14-31.05.06	22	13	3	0	16	4	14	0	18	34
102	2006	2	09.07.06	70	9	2	0	11	12	15	2	29	40
102	2007	1	27.05.07	27	7	4	0	11	13	11	1	25	36
102	2007	2	02.-03.07.07	63	7	6	0	13	5	18	1	24	37
108	2002	1	13.06.02	44	11	3	0	14	0	0	1	1	15
108	2002	2	02.07.02	63	3	2	0	5	5	2	5	12	17
108	2003	1	27.05.03	27	8	11	0	19	6	12	4	22	41
108	2003	2	30.06.03	61	15	10	0	25	3	7	1	11	36
108	2004	1	27.05.04	27	5	10	0	15	1	16	5	22	37
108	2004	2	17.07.04	78	7	13	0	20	6	10	2	18	38
108	2005	1	13.05.05	13	5	7	1	13	1	6	2	9	22
108	2005	2	01.08.05	93	4	7	0	11	3	12	5	20	31
108	2006	1	14.-21.05.06	20	11	5	0	16	3	11	5	19	35
108	2006	2	11.07.06	72	8	6	0	14	6	10	4	20	34
108	2007	1	27.05.07	27	16	9	0	25	5	6	1	12	37
108	2007	2	05.-07.07.07	66	12	9	0	21	6	12	3	21	42
111	2002	1	03.06.02	34	2	3	2	7	0	1	12	13	20
111	2002	2	21.06.02	52	2	3	0	5	1	5	9	15	20
111	2003	1	10.05.03	10	2	3	1	6	1	12	16	29	35
111	2003	2	06.07.03	67	1	4	0	5	2	16	17	35	40
111	2004	1	02.06.04	33	6	10	1	17	4	5	4	13	30
111	2004	2	20.-22.07.04	82	4	6	1	11	0	11	8	19	30
111	2005	1	22.05.05	22	8	30	1	39	4	9	4	17	56
111	2005	2	04.08.05	96	3	7	1	11	1	16	2	19	30
111	2006	1	13.-31.05.06	22	13	16	0	29	3	9	1	13	42

111	2006	2	16.-17.07.06	77	9	14	0	23	3	21	3	27	50
111	2007	1	30.05-03.06.07	31	0	7	1	8	2	13	8	23	31
111	2007	2	08.07.07	69	3	9	1	13	2	14	7	23	36
126	2002	1	15.06.02	46	4	3	2	9	3	4	13	20	29
126	2002	2	29.06.02	60	1	5	0	6	0	6	8	14	20
126	2003	1	11.-12.05.03	11	8	12	0	20	3	19	49	71	91
126	2003	2	18.06.03	49	5	17	1	23	4	6	16	26	49
126	2004	1	13.05.04	13	0	7	0	7	3	7	18	28	35
126	2004	2	13.07.04	74	4	15	1	20	1	15	14	30	50
126	2005	1	11.05.05	11	6	7	0	13	0	7	9	16	29
126	2005	2	04.08.05	96	0	4	0	4	6	8	13	27	31
126	2006	1	13.-31.05.06	26	12	9	0	21	6	9	1	16	37
126	2006	2	16.-17.07.06	77	6	14	0	20	7	17	4	28	48
126	2007	1	03.06.07	34	8	12	0	20	3	11	7	21	41
126	2007	2	03.07.07	64	4	7	0	11	7	13	13	33	44
134	2002	1	29.05.02	29	4	2	0	6	9	3	1	13	19
134	2002	2	15.07.02	76	1	5	0	6	3	4	6	13	19
134	2003	1	16.05.03	16	4	4	0	8	5	5	2	12	20
134	2003	2	25.06.03	56	5	12	0	17	8	16	4	28	45
134	2004	1	30.05.04	30	4	9	0	13	3	16	2	21	34
134	2004	2	12.07.04	73	1	14	0	15	0	8	0	8	23
134	2005	1	18.-20.05.05	20	2	7	0	9	3	18	1	22	31
134	2005	2	07.08.05	99	1	11	0	12	2	17	0	19	31
134	2006	1	07.-08.06.06	38	7	5	0	12	7	13	0	20	32
134	2006	2	22.07.06	83	5	12	0	17	6	16	0	22	39
134	2007	1	25.-26.05.07	26	2	11	0	13	7	10	0	17	30
134	2007	2	07.-16.07.07	75	8	9	0	17	5	11	1	17	34
138	2002	1	30.05.02	30	0	2	2	4	0	2	15	17	21
138	2002	2	13.06.02	44	0	2	0	2	0	3	9	12	14
138	2003	1	29.05.03	29	2	4	1	7	0	6	21	27	34
138	2003	2	10.07.03	71	0	4	1	5	1	2	38	41	46
138	2004	1	28.05.04	28	1	4	2	7	1	2	29	32	39
138	2004	2	24.07.04	85	0	2	2	4	0	3	26	29	33
138	2005	1	17.05.05	17	1	5	0	6	1	13	9	23	29
138	2005	2	03.08.05	95	0	3	1	4	0	12	17	29	33
138	2006	1	09.06.06	40	2	10	0	12	0	11	13	24	36
138	2006	2	21.-23.07.06	83	1	4	0	5	1	16	8	25	30
138	2007	1	24.05.07	24	1	7	1	9	0	20	6	26	35
138	2007	2	12.-15.07.07	74	1	12	1	14	2	26	6	34	48
Total					868	1085	86	2039	548	1354	1110	3012	5051

Appendix B.

AICc weights for different models of p in the 12 ponds keeping Φ constant.

Model	001	011	014	032	032A	089 [1)]	102	108	111 [1)]	126	134	138 [1)]	Mean
Φ(.) p(genotype*sex*time)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Φ(.) p(genotype*sex*season)	0.0039	0.0220	0.0081	0.0000	0.0000	0.0005	0.1028	0.0009	0.0264	0.0032	0.0403	0.0000	0.0173
Φ(.) p(genotype*sex)	0.3055	**0.3588**	0.0685	0.0001	0.0002	0.0002	0.0108	0.0729	0.0289	0.0152	0.0320	0.0027	0.0746
Φ(.) p(genotype*time)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Φ(.) p(genotype*season)	0.0024	0.0278	0.0984	0.0000	0.0001	0.0033	0.2302	0.0066	0.0374	0.0427	0.0181	0.0008	0.0390
Φ(.) p(sex*time)	0.0000	0.0241	0.0002	0.0005	0.0024	0.0471	0.0000	0.0000	0.0061	0.0011	0.0047	0.0002	0.0072
Φ(.) p(sex*season)	0.0877	0.1318	0.0331	0.0005	0.0002	0.0203	**0.2887**	0.0377	**0.7283**	0.2962	0.0054	0.0011	0.1359
Φ(.) p(genotype)	0.0366	0.0488	0.2446	0.0001	0.0008	0.0004	0.0050	0.1432	0.0495	0.0440	0.0097	0.0041	0.0489
Φ(.) p(sex)	**0.5082**	0.3188	0.0494	0.0004	0.0008	0.0016	0.0751	0.1423	0.0346	0.1169	0.0060	0.0023	0.1047
Φ(.) p(time)	0.0007	0.0043	**0.3520**	**0.9966**	**0.9947**	**0.8663**	0.0022	0.0650	0.0003	0.0031	**0.8618**	**0.9736**	0.4267
Φ(.) p(season)	0.0166	0.0380	0.0846	0.0013	0.0002	0.0584	0.2615	0.1410	0.0256	**0.4068**	0.0169	0.0088	0.0883
Φ(.) p(.)	0.0384	0.0258	0.0611	0.0006	0.0006	0.0020	0.0238	**0.3904**	0.0630	0.0709	0.0051	0.0065	0.0573

Grey marks AICc weights for models where the ΔAICc (data not shown) was <2.
Bold marks the best model per pond.
[1)] data includes LRR males in these ponds.

Appendix C.

AICc weights for different models of Φ in the 12 ponds, using the previously identified best models for p (2nd row).

	001	001	011	011	014	014	032	032A	089 [1)]	102	102	108	111 [1)]	126	134	138 [1)]
Model p ->	sex	geno*sex	geno*sex	sex	time	geno	time	time	time	sex*season	season	.	sex*season	season	time	time
Φ(geno*sex*time)	0.1031	**0.8970**	**1.0000**	1E-05	0	0	-	**1**	**1**	-	-	**0.9604**	**0.9998**	**1**	0	**0.9798**
Φ(geno*sex*season)	0	0	0	0	0.0006	0.0003	0.0010	0	0	0.0004	0.0005	0.0003	0	0	0.0172	0.0002
Φ(geno*sex)	0	0	0	0	0.0059	0.0022	0.0367	0	0	0.01173	0.0200	0.0157	0.00002	0	0.1382	0.0025
Φ(geno*time)	0	0	-	1E-05	0	0	0	7E-05	0	0.00015	9E-05	0.0010	0.00004	-	0.0926	0.0006
Φ(geno*season)	0	0	0	0	0.0195	0.0079	0.0115	0	0	0.00424	0.0059	0.0008	0	0	0.0419	0.0005
Φ(sex*time)	0	0	0	0	0	0.0001	0	0	0	0	0	0.0032	0	0	0	0
Φ(sex*season)	0	0	0	0	0.0237	0.0176	0.0890	0	0	0.01525	0.0223	0.0008	0.00003	0	0.0201	0.0007
Φ(geno)	0	0	0	0	0.0285	0.0098	0.1253	0	0	0.05014	0.0441	0.0134	0.00001	0	0.0472	0.0084
Φ(sex)	0	0	0	0	0.0355	0.0243	0.1455	0	0	0.08439	0.1451	0.0013	0.00002	0	0.1493	0.0040
Φ(time)	0	0	0	0	0.0003	0.4907	0.0017	0	0	0.00043	0.0006	4E-05	0	0	0.0001	0
Φ(season)	0	0	0	0	**0.1097**	0.0948	0.2478	0	0	0.09069	0.0793	0.0009	0.00002	0	0.1283	0.0011
Φ(.)	0	0	0	0	0.0759	0.0527	**0.3415**	0	0	**0.23927**	0.1854	0.0021	0.00006	0	**0.3651**	0.0023

Grey marks AICc weights for models where the ΔAICc (data not shown) was <2.
Bold marks the best model per pond.
"-" means that the analysis did not converge.
[1)] data includes LRR males in these ponds.
geno is an abbreviation for genotype

Later version published in Heredity, doi:10.1038/hdy.2010.37

The effects of geographic distance, sea barriers and ecology on the genetic structure and diversity of all-hybrid water frog populations

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Abstract

The history of population size and migration patterns leaves its mark in the genetics of populations. We investigate the genetic structure of the edible frog, *Pelophylax esculentus* in the Danish archipelago and adjacent countries. This frog is of particular interest because it is a hybrid that, in this area, forms all-hybrid populations of diploid (LR) and triploid (LLR and LRR) genomotypes with no (or only very few) adults of the parental species (LL and RR). The present study is the first to cover the entire geographic range of Danish, Swedish and German all-hybrid populations, documenting their extension and providing a broad picture of their diversity of neutral genetic markers and genomotype proportions. With 18 microsatellite markers, we demonstrate that genetic diversity declines northwards in agreement with the glacial refuge and central-marginal hypotheses; however populations on small and medium-sized islands are no less diverse than those on large islands and continental peninsulas. Isolation by distance exists across the archipelago, with limited influence of the fragmentation by brackish seawater. The extremely low genetic diversity in all-hybrid populations, compared to adjacent populations, might be responsible for the maintenance of their special breeding system. We also demonstrate large variation in the proportions of LLR, LR and LRR genomotypes between ponds, but little geographic pattern in their distribution. Instead, we found relationships between the proportions of these genomotypes and some of the 15 ecological pond parameters monitored. Size differences between LLR, LR and LRR further suggest ecological differences.

Introduction

Genetic structure arises by restrictions on population size and gene flow which lead to loss of genetic diversity, because finite populations loose genetic diversity due to genetic drift (Frankham 1996). Thus, genetic diversity is expected to decrease with distance from a glacial refuge as a consequence of sequential founder events (post-glacial colonization hypothesis) and/or from a species' centre to its periphery due to decreased density and increased isolation (central-marginal hypothesis, Eckert et al. 2008). Furthermore, mainland populations have been shown to be more diverse than island populations, where genetic diversity decreases with island size (Frankham 1996; 1997). In accordance with the island size effect, human-mediated habitat fragmentation reduces population size and connectivity world-wide, reducing neutral genetic diversity in many populations (Aguilar et al. 2008).

In a conservation perspective, genetic structure is of concern, as genetic variation is the prerequisite for adaption to new and changing environments (Frankham 1996; Willi et al. 2006). Even in stable environments, inbreeding depression can lower the fitness of small and isolated populations (Keller & Waller 2002; Reed & Frankham 2003). On the other hand, genetic structuring can lead to speciation, as evidenced by the much higher proportions of endemic species per area on islands than on the mainland (Kier et al. 2009).

The archipelago, adjacent peninsulas and continent of Denmark, South Sweden and North Germany offers the opportunity for studying the effect of natural fragmentation and island size on the genetic structure of low-mobility terrestrial and limnic species. This whole area was a coherent land mass 9200-7000 years ago, when the current herpetological fauna colonized the Scandinavia from the south or southeast (Fog et al. 1997). Postglacial, sequential founder events should thus be detectable as decreasing genetic diversity in a northern direction, whereas founder population sizes should be proportional to the present-day island sizes and not to their degree of isolation. Depending on the relative strength of genetic signals from, first, the dilution effect from the post-glacial migration and, second, the drift effect related to island size and isolation, both signals might be detectable in low-mobility species at microsatellite marker loci.

The low dispersive abilities of amphibians is among the characteristics that make them attractive subjects for population genetic studies, as they lead to population structure over relatively short distances (Beebee 2005). Another characteristic is that they presently experience dramatic declines worldwide (Stuart et al. 2004; Beebee 2005). The present study was made on the edible frog, *Pelophylax esculentus* (called *Rana esculenta* untill Frost et al. 2006). This frog is of particular interest to evolutionary biologists because of its hybrid origin

and bizarre reproductive mode. Moreover, Denmark, South Sweden and North Germany comprise the only known larger area, where these frogs form biologically unique all-hybrid populations of diploid and triploid hybrids, whereas the parental species do not survive (Graf & Polls Pelaz 1989; Günther & Plötner 1989-1990; Christiansen et al. 2005; Arioli 2007 chap. 3-4).

P. esculentus is widespread in Europe and is a hybrid between the pool frog, *P. lessonae* and the lake frog, *P. ridibundus*. The genomic constitution, here called genomotype (as in Bogart et al. 2007), of these parental species are denoted LL and RR, respectively. Hybrids employ different hybridogenetic modes of reproduction in different areas (reviewed by Graf & Polls Pelaz 1989). In most of Europe, *P. esculentus* frogs have the diploid genomotype LR, and reproduce hemiclonally, transmitting a clonal copy of only the R genome to gametes. They thus depend on backcrossing with *R. lessonae* to form a new generation of hybrids (L-E system). Inter-hybrid matings are usually futile, because the resulting RR offspring are homozygous for recessive deleterious mutations in the clonally transmitted R-genome (Vorburger 2001a; Guex et al. 2002). Modifications of this system include reproduction with *P. ridibundus* instead of *P. lessonae* in Eastern Europe (R-E system), triploid LLR and LRR individuals (can be present in all breeding systems) and the all-hybrid populations (E-E system).

Studies on gamete patterns in all-hybrid populations agree that triploid frogs of both sexes produce haploid gametes with the genome they have in double dose, whereas diploid frogs produce LR or R gametes (Graf & Polls Pelaz 1989; Jakob 2007 chap 5; Christiansen 2009). The great majority of LR gametes are eggs, as LR sperm is rare in most populations. Because the male-determining Y factor is not present in R genomes, LRR males are probably only formed when rare LR sperm fertilizes R eggs. This explains why LRR males are very infrequent in most ponds (Christiansen 2009).

Although reproduction in all-hybrid *P. esculentus* is now largely understood, two central questions about all-hybrid populations remain. The first question is how these populations remain all-hybrid. Various studies have shown that parental species (LL and RR) arise from hybrid x hybrid matings, but die off during larval stages (Christiansen et al. 2005; Arioli 2007 chap. 3). The inviability of RR offspring from hybrid x hybrid matings in the L-E system is explained with the diploid hybrids' clonal way of reproduction (Vorburger 2001a; Guex et al. 2002), but this explanation can not hold for all-hybrid populations where recombination between the two Ls occurs in LLR and between the two Rs in LRR frogs (Christiansen & Reyer 2009). An alternative hypothesis is that the genetic diversity in the L

and R genomes of all-hybrid populations is so low that, despite regular recombination within them, LL and RR offspring nevertheless become homozygous for a sufficient number of recessive deleterious mutations to kill them. To test this hypothesis, the genetic diversity of all-hybrid populations should be compared to that of adjacent, mixed populations.

The second question concerns why all-hybrid populations differ in genomotype proportions (LLR, LR and LRR). Several studies have documented differences in abundance of LLR, LR and LRR males and females between ponds (Christiansen et al. 2005; Jakob 2007 chap. 2). In contrast, models have shown that differently composed founder populations should all converge on the same equilibrium population structure, unless among-pond variation in the gametogenetic pattern or in selection on the various genomotypes is present (Som & Reyer 2006; Christiansen 2009). The relative importance of gamete patterns and ecology for shaping genomotype proportions are, however, not known. An analysis of the abundance of different genomotype proportions and their distribution over geographic areas and habitats would help to elucidate factors responsible for genomotype proportion differentiation.

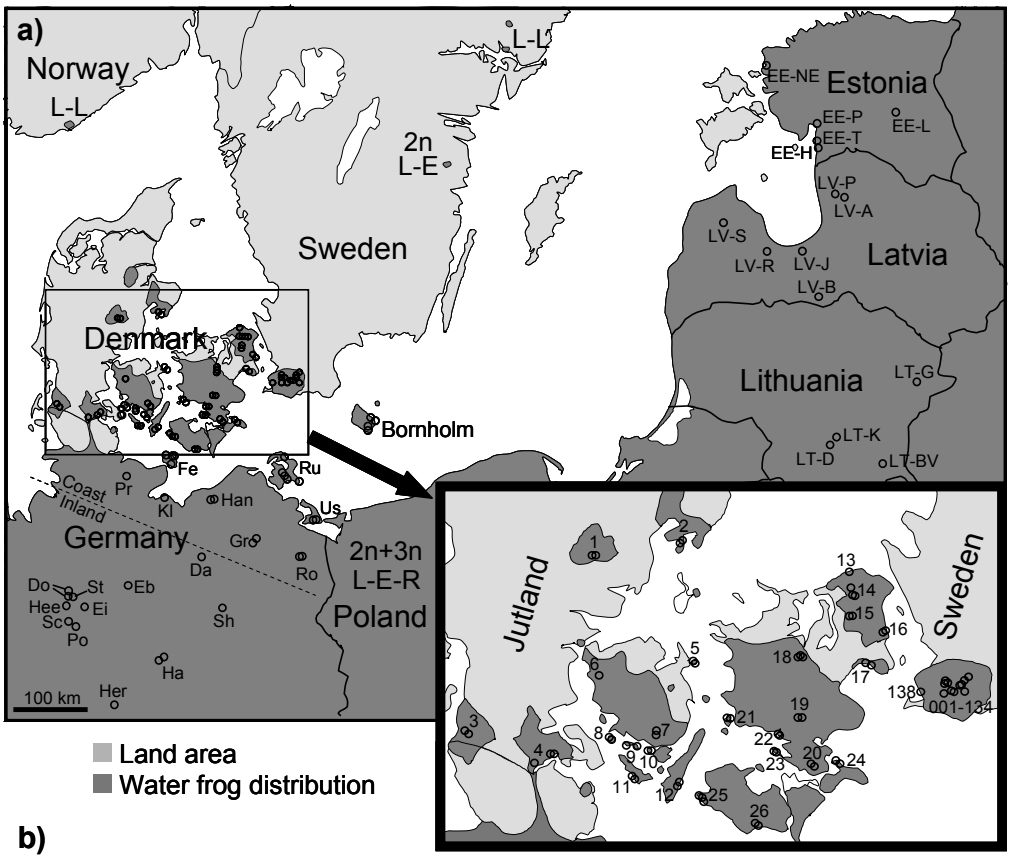
The present study is the first to cover the entire geographic range of Danish, Swedish and German all-hybrid populations, documenting their extension and providing a broad picture of their diversity of neutral genetic markers and genomotype proportions. Analyzing 13 L- and 13 R-specific microsatellite loci, we expected genetic diversity to increase: 1) In direction north and maybe also east for the R genome as *P. ridibundus* has its distribution centre southeast of the study area. 2) With land area, going from small over large islands to the mainland. 3) Going from all-hybrid to adjacent, mixed populations, in order to explain the inviability of LL and RR in the former. Moreover, we investigate population differentiation, isolation by distance and the effect of sea on isolation by distance. Finally, we relate proportions of the LLR, LR and LRR genomotypes to geography and ecology.

Materials and methods

Frog sampling

Frogs were collected in 118 ponds in six countries (Fig. 1 and Appendix). The frogs were caught at night by hand or dip net while dazzling them with a strong torch. The goal was to obtain at least 30 adult frogs per pond, including at least 10 adults of each sex, but this was not always accomplished (Appendix). All frogs had a finger tip cut off for DNA analysis and were returned to their home pond. Danish, Swedish and German frogs were also measured from snout to vent (with straight back) and sexed by the presence or absence of vocal sac

openings. Frogs with snout-to-vent lengths of ≥55 mm (≥50 mm for *P. lessonae*) were defined as adults. All ponds were sampled only once, except for the Swedish ponds which were sampled twice in 2005; recaptures were excluded from the second sample.



b)

Area	#Ponds	Year	Sex+length	Genetic data	Ecology data
Denmark[1]	54	2005	yes	genomo+allele	15 variables
Bornholm	4	2001	yes	genomo	none
Sweden*2[2]	12	2005	yes	genomo+allele	15 variables
Germany Coast	20	2001+04	yes	genomo	wood/open
Germany Inland	13	2005+07	yes	genomo+allele	wood/open
Baltic states	7	2004	no	genomo+allele	none
Baltic states	8	2004	no	genomo	none

Figure 1. Overview of a) pond locations and b) sampling scheme. Map based on Fog et al (1997), Rybacki and Berger (2001), Zeisset and Beebee (2001), Arioli (2007 chap. 5) and own information. L-L, L-E and L-E-R are breeding systems reported in the literature. [1] Denmark excl. Bornholm. [2] Swedish ponds were sampled twice, but to keep sample sizes relatively even in analyses of allele data, the second sample was only used in analyses of genomotype proportions. Baltic states = Estonia, Latvia and Lithuania.

Pond data

Geographic coordinates were obtained from Google Earth (Google 2009) and Det levende Danmarkskort (Kort & Matrikelstyrelsen 2002).

In Danish and Swedish ponds, 15 ecological parameters were measured between May and July 2005. Oxygen content, oxygen saturation, pH, connectivity and water temperature were measured at night, between 00:20 and 06:40, 10cm below the surface 0.5-2 m from the

shore with a HQ20 LDO sensor and a Hach sensIon pH electrode (Hach-Lange GmbH, Hegnau, Switzerland). The remaining 10 parameters were recorded by daylight. Surface area was calculated from the estimated length and width of the pond at the visit, taking the shape into account. Depth was measured as the maximum depth a fist-sized stone would sink to when lowered from a small rubber boat. Submerged/floating vegetation as well as emerging vegetation was scored as the percentage of the surface area covered. Bank shape was scored as 1 (flat), 2 (medium) or 3 (steep). The presence of short grass (cut or grazed), long grass, bushes/single trees, wood and agriculture within 100 meters of the ponds was each quantified on an ordinal scale from -100 to +100. Positive values describe the percentage of the pond periphery lined by the given land vegetation type while negative values described the distance in meters to the vegetation type, if it did not occur adjacent to the pond. Land vegetation types not present within 100m of the pond were given the score -100. Time and date of the registrations were noted.

German ponds were only categorized as open land, intermediate or wood ponds.

Genomotype determination

Frogs from all Danish, Swedish, Germany Inland and seven of the Baltic ponds were analyzed with the full set of 18 microsatellite primer pairs (Table 1). Extraction, PCR and sequencer protocols are described in Christiansen and Reyer (2009). However, instead of forward primer Ga1a19, forward primer Ga1a19redisigned was used (Christiansen 2009).

With very few exceptions (see results) all alleles encountered were specific to either the L or R genome (Table 1). Specificity was known for many alleles from previous studies (Christiansen 2005; 2009), confirmed in *P. lessonae* and *P. ridibundus* in the present study, and inferred for new alleles in *P. esculentus* on the basis of alleles with known specificity. Of the eight primer pairs that amplify both L and R specific alleles, four show dosage effect (Table 1). At each of these four loci, the relative intensity of L and R alleles amplified was used to distinguish LLR, LR and LRR frogs (Christiansen 2005). The final genomotype was determined as the genomotype indicated by the majority of these dosage effect loci, and the remaining loci were checked for congruence with this genomotype.

Whereas LLLR and LRRR tetraploids can be identified by extreme dosage effects, LLRR tetraploids will appear as LR diploids, if homozygous for all L and for all R loci. To estimate the magnitude of this potential error source, the probability of homozygosity at all L and at all R loci was, for each pond, calculated as the product of $1-H_E$ of all loci. Partly

heterozygous LLRR frogs should show LR dosage effect in combination with incongruent heterozygosity for some L and/or R loci.

All frogs from Germany Coast and Bornholm, and eight of the Baltic state ponds had their genomotypes determined by Martina Arioli and Christian Jakob on the basis of three to four of the dosage effect loci mentioned above. Allele data from these frogs were not used, as they did not comprise the full set of 18 loci. To keep sample sizes relatively constant in the genetic analyses, allele data from the second sample in Swedish ponds were also not used.

Table 1. Microsatellite allele length ranges, H_E in each genome, number of incongruent cases for each country (and number of ponds with >2 cases, where incongruent loci were not excluded but corrected) and the correction for incongruence.

Primer	L alleles	R alleles	H_E L	H_E R	DK**	SE**	D**	Bal**	Incongruence correction
Ca1b6*	74-90	83-96	0.109	0.476	1	1	23(3)	4(1)	L -> R (four different alleles)
Res16*	121+125	123+127	0.015	0.174	21(0)	7(0)	24(5)	1	R -> L (two different alleles)
RlCA1b5*	119+145	132+134	0.001	0.288	2	3(0)	2		
Ga1a19red.*	195+230	199-243	0.009	0.535	2	1	1		
RlCA2a34	113-156	106+109	0.666	0.033	9(1)		3(0)	17(2)	L -> null (DK), R -> null (Bal)
Re1CAGA10	90-98	95-185	0.041	0.797	2	1	3(1)		L -> null
Rrid013A	290-302	281+287	0.158	0.068		1	14(2)		L -> null
Rrid059Ared.	278	300-337	0.000	0.590			2		
Res20	85-151		0.547					2	
RlCA5	233-264		0.510		1	2	12(1)		L -> R
ReGA1a23	98-139		0.786		1				
RlCA1a27	95-141		0.717		1		1		
RlCA18	171-196		0.259						
Re2CAGA3		169-236		0.784	1		1	5(1)	R -> L
Rrid064A		211-235		0.649	256(9)		2		R -> null in all Jutland ponds
Res22		82-124		0.721			5(0)		
Rrid169A		176-203		0.567		6(1)	23(3)	12(2)	R -> null
Rrid135A		107-207		0.520					

* Dosage effect when the L and R alleles are not too different in length. Dosage effects were clearer with Ga1a19redesigned and Res16 than with Ca1b6 and, especially, RlCA1b5.
** Total sample sizes: DK (Denmark) 1562 individuals (54 ponds), SE (Sweden, first sample only) 409 (12), Bal (Baltic states) 224 (12) and D (Germany) 312 (13).

Allele data

The full allele genotype of frogs, for which allele data were obtained (Fig. 1b), was inferred on the basis of its overall genomotype. However, sometimes the number of alleles at one or few loci was, upon repeated analyses, incongruent with the overall genomotype. When a locus was incongruent in only one or two individuals in a pond, the phenomenon was deemed quantitatively unimportant and the locus was coded as missing data in these frogs. However, when a locus showed incongruence in more than two individuals in a pond, the phenomenon was attributed to either local genome inspecificity or to a null allele. In cases of local genome inspecificity, the allele in question was ascribed to the L genome in some frogs and to the R genome in other frogs in accordance with the overall genomotype. In case of a null allele, its

frequency was assessed in hemizygous individuals where non-amplification was not masked by other alleles. Assuming the same frequency of null alleles in hemizygous and non-hemizygous frogs, null alleles were randomly added to apparently homozygous frogs. This correction procedure should be unproblematic, as further analyses were only made on allele frequencies; not on observed homo- and heterozygosities.

Genetic parameters were calculated in SPAGeDi, version 1.2g (Hardy & Vekemans 2002), as this programme accepts a mixture of individuals with different ploidy levels. L and R genome data were split into separate input files, as SPAGeDi does not accept different ploidy levels in the same animal (in an LLR frog the L is diploid and the R is haploid). H_E (expected heterozygosity, i.e. genetic variability corrected for sample size, Nei 1978), F_{ST} (a measure of population differentiation, Weir & Cockerham 1984) and D_S (Nei's standard genetic distance, Nei 1978) were calculated for use in the analyses described below.

Analyses with allele data

Genetic diversity was measured as expected heterozygosity, H_EL and H_ER, and correlated with latitude, longitude and land area rank. Spearman rank correlations were used because most of the parameters were not normally distributed, and because of the difficulty to determine relevant land areas for peninsulas and continents. Land area ranks were based on land size and connectivity, so that Danish islands were ranked according to size (ranks 1-14, Danmarks Statistik 2009), followed by the peninsulas Sweden (rank 15) and Jutland (rank 16) and the continental German+Baltic areas (rank 17). Locality 23a+b was regarded an island and 9a and b separate islands. The tests were done in R, version 2.9.0 (R Development Core Team 2009).

Isolation by distance was investigated as the correlation between pairwise $F_{ST}/(1- F_{ST})$ and ln(km), according to Rousset (1997), and between D_S and ln(km). Pairwise geographic distances were calculated with the program Geographic Distance Matrix Generator (Ersts) from geographic coordinates after conversion to decimal degrees on the homepage of the Federal Communications Commission (www.fcc.gov/mb/audio/bickel/DDDMMSS-decimal.html). Correlations were tested with Mantel tests with 100,000 permutations in the program zt, version 1.1 (Bonnet & Van de Peer 2002). The influence of sea on isolation by distance was tested with partial Mantel tests (also with 100,000 permutations in zt) using a sea matrix with 0, 0.5 and 1 for ponds with a land connection, a bridge, or no connection, respectively, as a second explanatory variable. The relative proportions of total variance explained by the ln(km) and the sea matrix were calculated through ANOVAs in R.

Analyses with genomotype proportions

The samples were frequently biased towards one sex, maybe due to differential behaviour of males and females. Therefore, males and female genomotype proportions were treated separately in all analyses involving genomotype proportions.

For visualizing genomotype proportions, ternary plots (triangle diagrams) were drawn in the programme Past, version 1.91 (Hammer et al. 2001). Genomotype proportions were based on at least ten individuals of the same sex; smaller samples were not included in the diagrams.

To test whether genomotype proportion differences increased with geographic distance, pairwise differences in genomotype proportion between ponds were calculated as their Euclidian distances in the ternary plots and then correlated with ln(km) by Mantel tests, as described above. Ternary coordinates were converted into XY coordinates according to (Chen et al. 2007).

The effects of latitude and longitude on genomotype abundances were analyzed with logistic regressions in R, version 2.9.0 (R Development Core Team 2009). Logistic regressions handle proportional data taking sample sizes into account and thus make exclusion of small samples unnecessary. A model including latitude, longitude and their interaction was fitted separately to each genomotype within sex. As the data exhibited overdispersion with the binomial error distribution (residual deviation > the degrees of freedom), quasibinomial error distributions and F tests were used.

The analysis of the impact of ecological parameters on genomotype proportions was done on 62 Danish and Swedish ponds. Seven ponds (2a, 4z, 14a, 21b, 23a, 23b, 138) had to be excluded because of missing data, whereas “ponds” 17a and 25c were assemblages of 3 and 2 ponds, respectively, that were treated separately in the ecology analysis. Oxygen content, oxygen saturation, pH, connectivity and water temperature were corrected for both the time and date of measurement, as these were thought to have influenced the parameters. Correction was done by first converting time to decimal numbers after midnight and dates to the number of days after May 1st. Then residuals were extracted from a multiple regression of each parameter on time and date and their interaction. Surface area, depth, both types of pond vegetation and short and long grass were corrected for date in the same way.

To reduce the 15 potentially correlated variables to a smaller number of independent factors, they were subjected to a correlation matrix based principal component analysis (PCA; SYSTAT version 11) with an orthogonal varimax rotation. Principal components (PCs) with

an eigenvalue ≥ 1.00 were retained and tested for relations with genomotype proportions using logistic regressions with quasibinomial error distributions as described above.

The impact of forestation on German ponds was analyzed with logistic regressions with quasibinomial error distributions and with the categories open land, intermediate and wood as a continuous variable with values 1, 2 and 3, respectively. Swedish ponds were included in a second analysis, whereas too few Danish wood ponds had been sampled for inclusion of Danish ponds.

All means are given with ± standard deviation.

Results

Microsatellite data

All alleles could be assigned to either the L or R genome with low levels of incongruence (see Table 1 for microsatellite length ranges, H_E and incongruence). With 13 L and 13 R microsatellite loci analyzed in 2507 frogs, 476 cases of locus incongruence with the consensus genomotype were encountered, corresponding to only 1.05%. The majority of incongruent cases were German and Baltic, although the majority of samples were Danish and Swedish. In 11 ponds, incongruence was attributed to allele inspecificity; in 21 ponds to null alleles. Only one case of incongruence could possibly be attributed to tetraploidy. This adult frog from the Swedish pond 001 showed LR dosage at all dosage effect loci but showed heterozygosity for L alleles and homozygosity for an R allele at another locus. The probability of mistaking LLRR for LR, without signs of incongruence, was only 0.042 ±0.117, averaged over Danish, Swedish and German ponds (no polyploidy in Baltic ponds).

Breeding system distribution

54 Danish (excl. Bornholm), 12 Swedish and 33 German ponds were investigated in the supposedly all-hybrid area (Fig. 1 and Appendix). The 1457 adult Danish frogs analyzed were hybrids without exception. Of the Swedish frogs, 725 were hybrids while four (0.4%) were LL. The German sampling area included a bit of the transition zone from all-hybrid to other population types in the south, as the ponds Po, Ha2, Ha6 and Her had high proportions of parental species (71.4%, 12.5%, 33.3% and 63.0%, respectively). The remaining 30 German ponds were all-hybrid or almost so. In these ponds, 624 adult frogs were hybrid while three (0.5%) were LL. Thus, it was confirmed that the large area of Denmark, Sweden and North Germany is inhabited by all-hybrid populations.

Samples from eastern Bornholm were dominated by LRR among both males and females. Other genomotypes were LLR, LR and RR of both sexes (R-E system), however RR constituted only 0.45% of the males caught, as opposed to 26.1% of the females.

All frogs from the Baltic states were diploid, except from one LLL individual from EE-H. The northernmost water frog populations in the Baltic states were pure *P. lessonae*, as is also the case in Sweden (Fig. 1, Zeisset & Beebee 2001; Arioli 2007 chap. 5), Norway (Fig. 1, Zeisset & Beebee 2001) and a now-extinct native population in the United Kingdom (west of Denmark not in Fig. 1, Beebee et al. 2005). A little further south, *P. lessonae* and *P. esculentus* occurred together (diploid L-E system), whereas *P. ridibundus* only occurred in samples from Riga (LV-J) and southwards.

Genetic diversity

Genetic diversity was investigated in Denmark, Sweden, Germany Inland and half of the Baltic ponds, where the full microsatellite data set was available (Fig. 2 and Appendix). However, H_ER could only be calculated for one Baltic pond. Within the all-hybrid ponds, H_E in the L genome ranged from 0.000 to 0.351; mean 0.157 ±0.074. In the R genome, H_E was generally higher, ranging from 0.000 to 0.521; mean 0.186 ±0.117. Outside the all-hybrid area, H_EL averaged 0.315 ±0.129 in four German ponds, and 0.286 ±0.122 in seven Baltic ponds (Fig. 2). H_ER averaged 0.453 ±0.060 in the four German ponds, and was 0.615 in the Baltic pond. Genetic diversity was thus lower in all-hybrid populations than in the adjacent populations, but not significantly so (the confidence intervals of 1.96*S.D. overlap). In all-hybrid ponds, H_E was highly significantly positively correlated between the L and R genomes, indicating that although they are propagated independently, processes like founder effect and drift affect them similarly (Spearman rank two-tailed test on all-hybrid ponds, $r_s = 0.534$, n = 75, P = 8.06e-07).

As expected, both H_EL and H_ER correlated significantly and negatively with latitude (north) within all-hybrid ponds (n = 75, Spearman rank two-tailed tests, H_EL: $r_s = -0.557$, P = 2.10e-07 and H_ER: $r_s = -0.384$, P = 0.0007). This result was not confounded with the effect of land area, as latitude and the land area rank were not correlated ($r_s = -0.007$, P = 0.953). In the Baltic ponds, H_EL showed a steep negative relation with latitude, but due to the low sample size, this relation was not significant ($r_s = -0.643$, n = 7, P = 0.139).

Correlations between H_E and longitude (east) was not significant for any of the genomes within all-hybrid ponds (n = 75, H_EL: $r_s = -0.098$, P = 0.404 and H_ER: $r_s = 0.012$, P = 0.916). The correlation was almost significant for the R genome in the pooled

Danish+Swedish ponds (n = 66, r_s = 0.219, P = 0.078), but not in Germany Inland ponds (n = 13, r_s = 0.060, P = 0.842).

Also contrary to expectations, there were no significant positive correlations between genetic diversity and land area in all-hybrid populations (n = 75, H_EL: r_s = 0.120, P = 0.307; H_ER: r_s = 0.160, P = 0.171). Within Danish+Swedish ponds pooled, there was even a significantly negative correlation for the L genome (n = 66, r_s = -0.295, P = 0.016). It thus appears that small and especially medium-sized Danish islands have healthy water frog populations with high genetic diversity compared to large islands and, in particular, the Danish peninsula Jutland (Fig. 2).

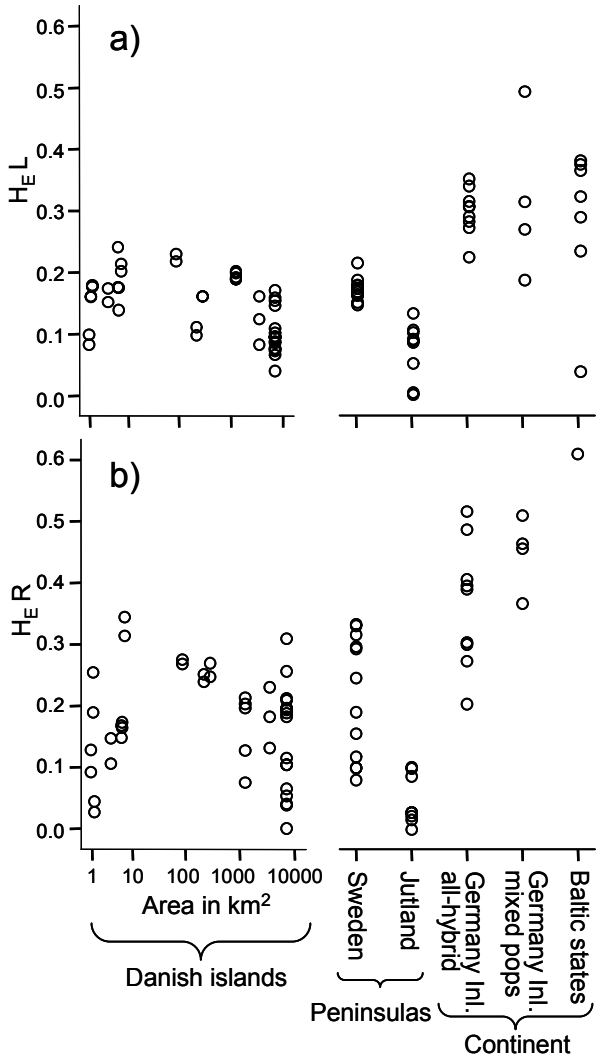


Figure 2. Genetic diversity, H_E, per pond in a) the L genome and b) the R genome. The x axis reflects increasing land area ranks based on land size and connectivity; however, within the "continent", all four categories have the same land area rank (see Methods).

F_{ST} and isolation by distance

Global F_{ST} was 0.455 for the L genome and 0.580 for the R genome; similar to the values for the subset of all-hybrid ponds (L: 0.451 and R: 0.598).

In tests for isolation by distance, D_S increased significantly with ln(km) in both genomes (Mantel tests on all ponds, L: r = 0.502, P = 0.00001; R: r = 0.363, P = 0.00001; Fig. 3). Partial mantel tests including the sea matrix were also highly significant for D_S (L: r = 0.461, P = 0.00001; R: r = 0.280, P = 0.00001). However, whereas 34.2% of the total variance in the L genome was explained by ln(km), only 0.3% was additionally explained by the sea matrix. For the R genome, the corresponding values were 16.0% and 3.3%. Explanations for the low explanatory power of the sea matrix include high correlation between the geographic distance and the presence of sea between ponds (Mantel test, r = 0.403, P = 0.00001), and that only 19% of the ponds were connected by land.

$F_{ST}/(1-F_{ST})$ gave less significant isolation by distance results than Ds; for the R genome isolation by distance was not even significant (Mantel tests: L: r = 0.189, P = 0.002; R: r = -0.006, P = 0.448. Partial mantel tests with sea matrix: L: r = 0.151, P = 0.019; R: r = -0.034, P = 0.257). The proportions of the variance explained by the geographic distance, and, aditionally, by the sea matrix were only 3.7% and 0.5%, respectively for the L genome and 0.2% and 0.7%, respectively, for the R genome.

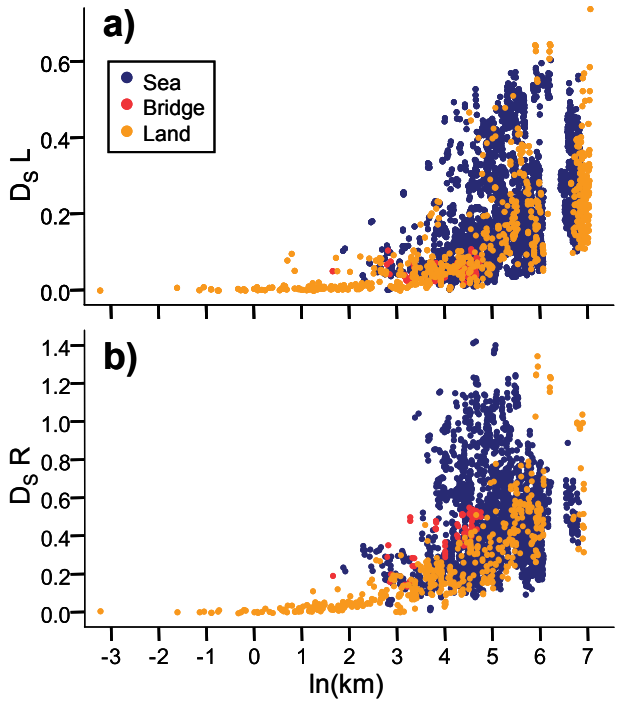


Figure 3. Isolation by distance in A) the L genome and B) the R genome. Colour indicates connectivity between the pairwise compared ponds.

Genomotype proportions in all-hybrid populations

On average, the all-hybrid populations sampled had 57.5% LLR, 33.9% LR and 8.2% LRR among males, and 17.4% LLR, 52.8% LR and 29.8% LRR among females. In addition, 0.38% LL was found among males and 0.07% among females. The proportions of the hybrid

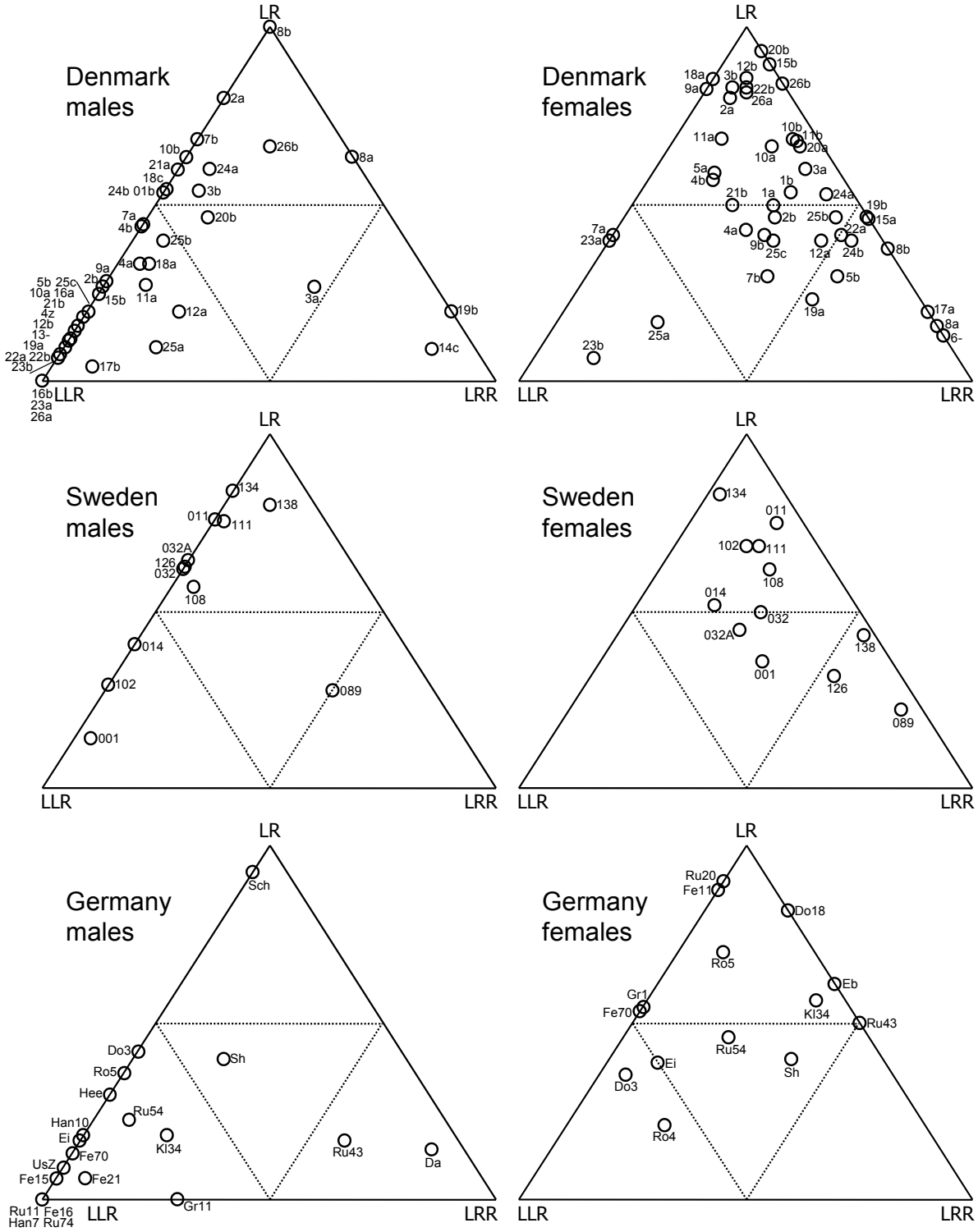


Figure 4. Proportions of LLR, LR and LRR within males and females in Danish, Swedish and German ponds with all-hybrid populations of *P. esculentus*.

genomotypes showed large variation between ponds (Fig. 4), forming a continuum rather than distinct population types.

Male and female genomotype distributions were very different, mainly because LRR were rare among males but common among females throughout the distribution area of the

all-hybrid populations (Fig. 4). The few ponds found with high LRR proportions among males were geographically scattered.

LLR female proportions decreased significantly in direction north and LLR and LR male proportions increased southeast and northwest, respectively (Table 2). Otherwise there were no significant relationships between genomotype proportions and latitude, longitude or their interaction.

Table 2. GLMs for males (n = 95 ponds, df = 91) and females (n = 93 ponds, df = 89)

	Res.	North		East		Interaction	
	dev.	F	P	F	P	F	P
mLLR	566	0.952	0.332	0.586	0.446	6.109	0.015*
mLR	440	1.344	0.249	0.981	0.325	9.716	0.002**
mLRR	401	0.002	0.967	0.022	0.882	0.058	0.811
fLLR	296	5.851	0.018*	0.314	0.577	0.857	0.357
fLR	316	0.029	0.866	0.269	0.606	0.148	0.701
fLRR	410	2.867	0.094	0.008	0.928	0.105	0.746

Pairwise differences in genomotype proportions between ponds increased significantly with geographic distance for males (Mantel test, n = 77 ponds, r = 0.075, P = 0.035), but not for females (n = 69 ponds, r = -0.015, P = 0.378). From Fig. 5 (shaded area) it seems that pairwise differences in genomotype proportions increased with geographic distance for both sexes up to approximately 3 ln(km) ~ 20 km, whereafter they could not increase further.

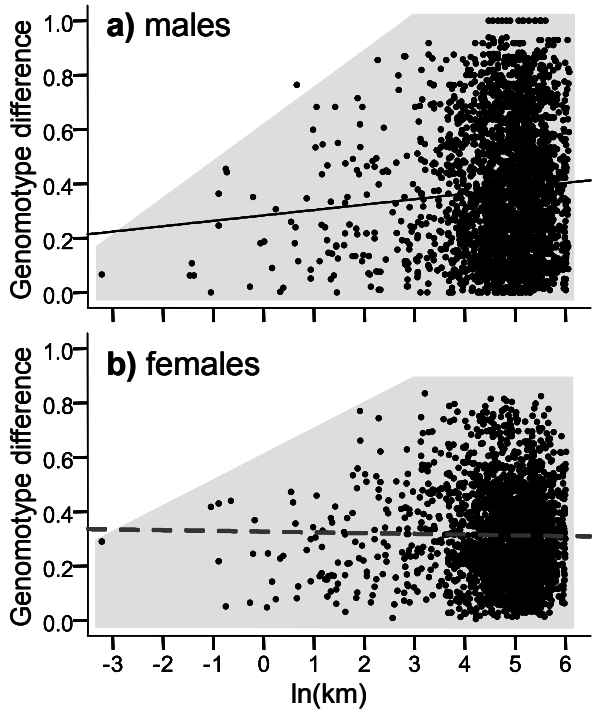


Figure 5. Pairwise differences in genomotype composition of Danish, Swedish and German all-hybrid populations as a function of geographic distance for A) males and B) females. Shaded area: see text. Ponds with less than 10 individuals sampled per sex were excluded. Therafter, the male data set contained 77 ponds and the female data set 69 ponds.

Genomotype versus ecology and body size

Principal component analysis of 15 ecological parameters gave six principal components (Table 3). Of these, PC2 (oxygen content, oxygen saturation, water temperature) was significantly negatively related to LR female proportions and significantly positively related to LRR proportions of both sexes. PC4 (-emerging vegetation, surface area, agriculture) was highly significantly negatively related to LRR female proportions, and PC5 (short grass, -long grass) was significantly negatively related to LLR female proportions (Table 4). The remaining PCAs were unrelated to genomotype proportions.

Table 3. Rotated Loading Matrix from PCA of ecological factors.

Parameter	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	2.819	2.566	1.860	1.654	1.526	1.106
Conductivity	0.911	-0.142	0.102	0.000	0.107	-0.157
pH	-0.910	0.140	-0.102	-0.003	-0.113	0.160
Submerged veget.	-0.622	-0.377	0.037	0.196	0.028	-0.298
O_2 saturation	-0.167	0.917	-0.022	0.025	0.088	0.042
O_2 content	-0.171	0.910	0.009	-0.026	0.130	0.005
Temperature	0.215	0.704	0.099	0.093	-0.024	0.084
Bank shape	-0.133	0.084	0.792	-0.074	-0.027	-0.115
Bushes/trees	0.190	-0.013	0.774	-0.090	0.221	0.103
Depth	0.197	0.014	0.607	0.367	-0.143	-0.094
Emerging veget.	0.272	-0.044	-0.212	-0.783	-0.075	0.061
Surfase area	0.411	0.140	-0.318	0.680	-0.021	-0.135
Agriculture	-0.141	-0.108	-0.151	0.578	0.019	0.617
Long grass	0.081	0.140	-0.125	0.055	0.910	0.090
Short grass	-0.091	-0.030	-0.214	0.011	-0.895	0.209
Wood	0.093	-0.157	0.007	0.160	0.088	-0.824

Table 4. GLMs for males (m) and females (f). n = 61 ponds, df = 54.

		PC1		PC2		PC3		PC4		PC5		PC6	
Genomo	Res.dev.	F	P	F	P	F	P	F	P	F	P	F	P
mLLR	294	0.061	0.806	3.015	0.088	0.328	0.569	0.644	0.426	0.0001	0.993	1.238	0.271
mLR	228	0.101	0.752	0.468	0.497	2.767	0.102	3.544	0.065	0.495	0.485	0.142	0.708
mLRR	195	0.003	0.956	4.405	0.041*	2.568	0.115	1.943	0.169	1.450	0.234	3.847	0.055
fLLR	113	1.2E-05	0.997	0.285	0.596	0.834	0.365	2.328	0.133	5.709	0.020*	3.363	0.072
fLR	188	0.337	0.564	4.598	0.037*	1.142	0.290	3.768	0.057	1.034	0.314	0.056	0.814
fLRR	220	0.327	0.57	5.798	0.019*	0.318	0.575	7.919	0.007**	0.073	0.789	1.662	0.203

In German ponds, LLR female proportions increased significantly with wood (logistic regression, df = 29, F = 5.899, P = 0.022) and LRR proportions of both sexes decreased significantly with wood (males: df = 31, F = 5.456, P = 0.026; females: df = 29, F = 5.753, P = 0.023). When including Swedish samples (12 ponds but more individuals per pond), only the decreasing relationship between LRR male proportions and wood remained significant (df = 43, F = 6.621, P = 0.014).

The mean lengths of LLR, LR and LRR in all-hybrid ponds increased in the order LLR<LR<LRR with males generally being smaller than females (mLLR: 65.5 ±5.0, mLR: 66.2 ±6.0, mLRR 73.9 ±8.2, fLLR: 70.5 ±7.7, fLR: 73.1 ±8.8 and fLRR: 75.0 ±9.7; Spearman rank two-tailed tests, males: $r_s = 0.186$, n = 1237, P = 4.11e-11; females: $r_s = 0.160$, n = 1249, P = 1.42e-08). However, LRR males were larger than both LLR and LR females. Because in mixed populations of diploid hybrids (LR) and parental species (LL, RR) size differences are related to ecological differences, the increase in size from LLR to LRR also suggests ecological differences between the genomotypes, with most differentiation between LLR and LRR.

Discussion

Genetic diversity and fragmentation

Our study on all-hybrid populations of the edible frog confirms the expectation that genetic diversity declines towards the northern distribution limit. This was to be expected for two reasons that cannot be disentangled: both postglacial sequential founder events and the central-marginal model predict that higher isolation and lower effective population size decrease genetic diversity at a species' range (Eckert et al. 2008). However, although H_E values depend on the markers used, so that comparisons between studies and species are problematic, the mean H_E values of 0.157 for the L genome and 0.186 for the R genome found in all-hybrid populations appear exceptionally low compared to other studies. At similar or higher latitudes, considerably higher H_E values were found in tree frogs (0.58, Denmark, Andersen et al. 2004), natterjack toads (0.319, Denmark, Allentoft et al. 2009), common frogs (0.614, Denmark, Sweden, Norway, Finland, Palo et al. 2004) and moor frogs (0.431, Denmark, Sweden Finland, Knopp & Merila 2009). Only the small and very isolated Norwegian and Swedish *P. lessonae* populations (Fig. 1) were reported to have lower H_E values, as no variation was found at six microsatellite loci (Zeisset & Beebee 2001). Further south, *P. lessonae* had higher H_E values (0.428, Central Europe, Zeisset & Beebee 2001; 0.424, Belgium, Holsbeek et al. 2009), and so had *P. ridibundus* (0.428, UK and Hungary, Zeisset & Beebee 2003; 0.588, Belgium, Holsbeek et al. 2009). This demonstrates that these parental species are not always genetically depleted.

Correlation between genetic diversity and land area has been shown in general (Frankham 1996) as well as for moor frogs in almost the same study area as ours (Knopp et al. 2007). In the present study, large islands, and especially the peninsula of Jutland, had unexpectedly low levels of genetic diversity compared to small and especially medium-sized

islands. As low diversity at neutral markers does not necessarily imply low variability at coding genes under selection (Reed & Frankham 2001), it cannot be predicted whether these populations will suffer from their low genetic diversity. However, it can be concluded that populations on even small islands are not particularly threatened by drift, as they are not more genetically depleted than populations elsewhere. The less intensive agriculture, and hence better habitat quality, of small islands might to some extend compensate for their small size by allowing large, dense frog populations. In accordance with this, agriculture has been shown to affect population size and genetic diversity negatively in common frog populations in southern Sweden (Johansson et al. 2005).

The present study found extremely high levels of population differentiation (F_{ST} = 0.455 for the L genome and 0.580 for the R genome). Explanations include the large geographic area relative to the dispersive abilities of the frogs and the low microsatellite diversity (Neigel 2002).

D_S showed much more isolation by distance than F_{ST}. This difference is possibly because D_S takes mutations into account, which are likely of importance given the several thousand years since colonization and fragmentation of the area. With D_S, isolation by distance was found across the entire area sampled, although 81% of the ponds were separated by sea. Two explanations are possible for this apparently small sea influence. 1) The brackish sea water is no large barrier to *P. esculentus* dispersal. In support of this, fishermen in the inner Danish waters sometimes catch *P. esculentus* when they set fish traps close to land (Fog et al. 1997). It can also not be excluded that people occasionally transport eggs, tadpoles or frogs between islands for hobby purposes. 2) The populations in the area have not yet reached migration-drift equilibrium after the fragmentation of the land area started about 7000 years ago. At least on the large islands and the continent, the frog populations are likely to be large, and in large populations drift is small so that equilibrium is only very slowly approached. Two previous studies on frogs in archipelagos show diverging results. One study found high levels of gene flow in the common toad and the common frog in a Finnish archipelago where the water is even more brackish than the present study (Seppa & Laurila 1999). The second study found strong differentiation on Panamanian islands in the strawberry frog, i.e. a terrestrial frog in an ocean archipelago (Rudh et al. 2007).

All-hybrid distribution

In the present study, all-hybrid populations were found as far south as ponds Sc and Sh (Fig. 1). Thus, they extend beyond, latitudes where another study found high levels of *P. lessonae*

(near Serrahn between Da and Gr, Berger & Günther 1988). A third study found high numbers of *P. ridibundus* south of ponds Sh and east of Ha and Her, supporting the conclusion from the present study that the all-hybrid area ends this far south (near Steckby, Berger & Günther 1991-1992).

Three things complicate the determination of the distribution of all-hybrid populations. First, the length used for defining adults is critical, as the frequency of non-hybrids decreases steeply with their size/age. While adult males can be identified by the presence of vocal sac openings, adult females not carrying eggs differ from juveniles of both sexes only by their size. Second, detection of rare non-hybrids is strongly dependent on sample size. Third, how long non-hybrids survive can depend on the weather conditions of particular seasons and can thus vary temporally (Vorburger 2001b). Hence, without established standard methods, the decision to call a population all-hybrid entirely depends on individual authors.

All-hybrid genomotype proportion differences

A continuum of genomotype proportions was found, so that no clearly differentiated population categories were identified. Genomotype proportions were very variable on a small geographic scale, leaving less variation to be explained by geographic variation on a regional scale.

In stead, we found some evidence for an effect of ecology on genomotype proportions, in accordance with the phenotypic effect of genome dosage observed on body length (LLR<LR<LRR within both sexes). Consistent for both sexes, LRR proportions increased with PC2, representing oxygen content and saturation and, with less weight, water temperature. This corresponds well with literature statements that oxygen is an important and critical parameter for RR that usually live and breed in large, well oxygenated water bodies and hibernate aquatically (Plenet et al. 2000a; Plenet et al. 2000b; Plötner 2005). The significant decrease of LR female proportions with PC2 can be seen as a logic consequence of the increase of LRR female proportions, as LLR females were generally infrequent. In males, both LLR and LR were much more common than LRR so that significant decreases in the former genomotypes could not necessarily be expected. Nevertheless, LLR males showed a strong negative trend with PC2. Water temperature has been found to increase the performance and ratio of LL to LR tadpoles (Negovetic et al. 2001) in accordance with the fact that warm water can hold less oxygen than colder water. Why oxygen content and water temperature were not negatively correlated in the present study is difficult to explain. Although no explanations are at present available for the correlations of certain genomotypes

with vegetation parameters (PC4, PC5 and wood), the present study thus adds to the data suggesting that ecology affects the proportions of LLR, LR and LRR (c.f. Peter et al. 2002; Jakob 2007 chap. 3).

How to remain all-hybrid?

The low genetic diversity within in the L and the R genome in all-hybrid *P. esculentus*, compared to adjacent populations (although too few samples for significance) and other amphibians (see above) appears to be compensated by high genetic heterozygosity in individual hybrids. As almost all alleles were genome-specific, every individual and pond had an overall expected heterozygosity of one, preventing inbreeding even in very small and homogeneous populations. Moreover, even if little genetic diversity is available for selection, the same genetic material can give rise to three different phenotypes due to dosage effect in di- and triploid individuals (LLR, LR and LRR). Although most genomotypes are required for reproduction, genomotype proportions and not just gene frequencies can quickly become adapted by natural selection.

Not only is low genetic diversity tolerable for the all-hybrid populations, it also seems to be the precondition for the inviability of parental species and thus for the existence of the all-hybrid populations in Denmark, South Sweden and North Germany. Whereas single ponds with all-hybrid frog populations in other parts of Europe (references in Christiansen et al. 2005) can possibly be explained by ecological conditions being optimal for *P. esculentus* and suboptimal for the parental species, the large all-hybrid area of Denmark, South Sweden and North Germany encompasses a range of habitats, some of which should be suitable for genetically healthy *P. lessonae* and/or *P. ridibundus*.

In spite of increasing isolation towards species' edges, differentiation rarely takes place here, because asymmetric gene flow from the larger central to the smaller peripheral populations counteracts local adaption in the smaller peripheral populations (Lenormand 2002; Eckert et al. 2008). All-hybrid populations of *P. esculentus* might, nevertheless, represent a special and rare example of differentiation towards the species edge.

The *P. esculentus* system is outstanding among hybridogenetic taxa (four fishes, one stick insect and two more water frog complexes involving *P. ridibundus*, references in Christiansen & Reyer 2009; Holsbeek & Jooris 2009) for its great variability with three breeding systems. The present study shows that also within the all-hybrid system there is a great variety of genomotype proportions. *P. esculentus* thus appears to be a great natural experiment with potential exciting outcomes if given time and space to proceed. Swedish all-

hybrid populations are currently expanding their range (Fog et al. 1997; Arioli 2007 chap 4). In Denmark, we got the clear impression from county offices and field herpetologists that the Danish distribution area has shrunk since the last description by Fog et al (1997), but that *P. esculentus* is thriving in areas where ponds have been dug to promote endangered amphibian species.

Acknowledgements

We are grateful to Lars Iversen, Eline Embrects, Kåre Fog and Egidijus Sireika for help with sampling and to many Danish, Swedish, German and Baltic colleagues for recommending sampling localities. Thanks, also, to many authorities and land owners for permissions to catch frogs. Furthermore, our gratitude goes to Sandra Röthlisberger for extraction and PCR, and to Christian Jakob and Martina Arioli for providing genomotypes for some of the frogs. Lukas Keller, Peter Wandeler, Christoph Vorburger and Josh Van Buskirk kindly helped with statistical advice; Christian Mayer was a good discussion partner. The study was supported by the Swiss National Fund (grant no. 31-64004.00 to H-U Reyer).

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Appendix

Ponds sampled. Genetic samples sometime included juveniles or unsexed adults not listed under males or females. H_E = expected heterozygosity.

				Males > 55mm (LL>50)						Females > 55mm (LL>50)						Genetic samples				
Pond	Country	Latitude	Longitude	LL	LLR	LR	LRR	RR	n_m	LL	LLR	LR	LRR	RR	n_f	n_L	H_EL	n_R	H_ER
All-hybrid populations																			
1a	Denmark, Jutland	56°02'54"	09°40'29"	0	2	1	0	0	3	0	3	8	5	0	16	24	0.085	24	0.102
1b	Denmark, Jutland	56°02'48"	09°39'28"	0	7	8	0	0	15	0	2	8	5	0	15	39	0.132	35	0.100
2a	Denmark, Jutland	56°09'03"	10°31'57"	0	3	12	0	0	15	0	2	12	1	0	15	35	0.003	31	0.028
2b	Denmark, Jutland	56°07'34"	10°32'28"	0	11	4	0	0	15	0	3	7	5	0	15	44	0.000	35	0.022
3a	Denmark, Jutland	54°56'45"	08°50'47"	0	4	4	7	0	15	0	1	9	5	0	15	35	0.051	42	0.028
3b	Denmark, Jutland	54°57'46"	08°49'27"	0	5	7	1	0	13	0	2	15	1	0	18	38	0.101	33	0.087
4a	Denmark, Jutland	54°55'42"	09°35'06"	0	13	7	1	0	21	0	4	6	4	0	14	52	0.087	40	0.016
4b	Denmark, Jutland	54°55'38"	09°33'60"	0	9	7	0	0	16	0	4	8	2	0	14	43	0.105	32	0.028
4z	Denmark, Jutland	54°51'54"	09°23'42"	0	12	2	0	0	14	0	4	5	0	0	9	39	0.090	23	0.000
5a	Denmark, Romsø	55°30'56"	10°47'08"	0	8	0	0	0	8	0	6	13	3	0	22	44	0.161	33	0.254
5b	Denmark, Romsø	55°30'38"	10°47'44"	0	8	2	0	0	10	0	3	6	11	0	20	41	0.160	41	0.189
6	Denmark, Funen	55°22'41"	09°55'59"	0	0	2	0	0	2	0	0	3	20	0	23	25	0.083	45	0.131
7a	Denmark, Funen	55°06'10"	10°30'04"	0	10	8	0	0	18	0	7	5	0	0	12	47	0.124	30	0.230
7b	Denmark, Funen	55°07'09"	10°30'30"	0	6	13	0	0	19	0	3	3	4	0	10	38	0.161	33	0.182
8a	Denmark, Lyø	55°02'50"	10°09'15"	0	0	7	4	0	11	0	0	3	16	0	19	30	0.175	50	0.173
8b	Denmark, Lyø	55°02'38"	10°09'25"	0	0	14	0	0	14	0	0	6	10	0	16	30	0.139	40	0.164
9a	Denmark, Avernakø	55°01'26"	10°15'09"	0	10	4	0	0	14	0	3	14	0	0	17	44	0.241	31	0.148
9b	Denmark, Avernakø	55°00'44"	10°19'56"	0	3	1	0	0	4	0	3	5	4	0	12	22	0.176	20	0.168
10a	Denmark, Hjortø	54°57'52"	10°29'30"	0	9	2	0	0	11	0	2	12	4	0	18	40	0.099	33	0.092
10b	Denmark, Hjortø	54°57'43"	10°29'09"	0	4	7	0	0	11	0	1	13	5	0	19	35	0.083	35	0.128
11a	Denmark, Ærø	54°52'18"	10°20'14"	0	7	3	1	0	11	0	4	13	2	0	19	41	0.218	33	0.275
11b	Denmark, Ærø	54°51'29"	10°23'33"	0	2	6	0	0	8	0	1	15	6	0	22	33	0.230	36	0.268
12a	Denmark, Langeland	54°50'33"	10°44'60"	0	9	3	3	0	15	0	2	6	7	0	15	41	0.161	40	0.247
12b	Denmark, Langeland	54°50'46"	10°45'05"	0	14	2	0	0	16	0	1	12	1	0	14	45	0.161	31	0.269
13	Denmark, Sealand	56°05'21"	12°08'38"	0	15	2	0	0	17	0	0	1	0	0	1	33	0.159	18	0.000
14a	Denmark, Sealand	55°58'53"	12°12'15"	0	5	2	2	0	9	0	0	2	3	0	5	19	0.154	19	0.115
14b	Denmark, Sealand	55°58'09"	12°13'42"	0	0	1	5	0	6	0	0	4	2	0	6	12	0.077	19	0.188
14c	Denmark, Sealand	55°58'03"	12°13'47"	0	2	2	17	0	21	0	0	0	6	0	6	29	0.066	50	0.193
15a	Denmark, Sealand	55°50'22"	12°14'26"	0	4	3	0	0	7	0	0	6	7	0	13	24	0.094	27	0.104
15b	Denmark, Sealand	55°50'11"	12°12'44"	0	9	3	0	0	12	0	0	17	2	0	19	40	0.040	33	0.065
16a	Denmark, Sealand	55°47'60"	12°32'55"	0	9	2	0	0	11	0	0	8	1	0	9	29	0.086	21	0.104
16b	Denmark, Sealand	55°48'04"	12°34'42"	0	19	0	0	0	19	0	0	0	0	0	0	38	0.095	19	0.053
17a	Denmark, Sealand	55°37'05"	12°27'17"	0	0	1	0	0	1	0	0	4	16	0	20	21	0.171	37	0.040
17b	Denmark, Sealand	55°37'00"	12°29'32"	0	20	1	2	0	23	0	1	2	4	0	7	51	0.109	36	0.038
18a	Denmark, Sealand	55°33'13"	11°53'47"	0	9	5	1	0	15	0	2	12	0	0	14	40	0.088	30	0.196
18b	Denmark, Sealand	55°33'14"	11°53'06"	0	5	2	0	0	7	0	2	6	1	0	9	23	0.076	17	0.209
18c	Denmark, Sealand	55°33'19"	11°52'50"	0	5	6	0	0	11	0	0	5	0	0	5	21	0.096	16	0.195
19a	Denmark, Sealand	55°17'23"	11°50'18"	0	9	1	0	0	10	0	4	4	9	0	17	40	0.146	36	0.309
19b	Denmark, Sealand	55°18'53"	11°41'31"	0	0	3	12	0	15	0	0	7	8	0	15	30	0.095	50	0.256
20a	Denmark, Sealand	55°00'31"	12°00'17"	0	4	1	1	0	6	0	1	14	6	0	21	32	0.073	34	0.182
20b	Denmark, Sealand	55°00'15"	12°01'12"	0	6	7	2	0	15	0	0	14	1	0	15	36	0.102	33	0.212
21a	Denmark, Agersø	55°13'29"	11°11'12"	0	4	6	0	0	10	0	0	5	3	0	8	22	0.214	21	0.344
21b	Denmark, Agersø	55°13'34"	11°10'46"	0	16	3	0	0	19	0	5	9	4	0	18	58	0.202	41	0.314
22a	Denmark, Enø	55°10'06"	11°40'06"	0	12	1	0	0	13	0	1	5	6	0	12	38	0.152	31	0.147
22b	Denmark, Enø	55°09'55"	11°40'01"	0	12	1	0	0	13	0	1	10	1	0	12	38	0.174	26	0.106
23a	Denmark, Knudsh. Odde	55°04'37"	11°39'15"	0	16	0	0	0	16	0	6	4	0	0	10	48	0.179	26	0.027
23b	Denmark, Knudsh. Odde	55°04'38"	11°39'14"	0	14	1	0	0	15	0	12	1	2	0	15	56	0.177	32	0.044
24a	Denmark, Møn	55°03'01"	12°16'03"	0	5	9	1	0	15	0	1	9	7	0	17	38	0.098	40	0.239
24b	Denmark, Møn	55°02'05"	12°16'58"	0	7	8	0	0	15	0	1	6	8	0	15	38	0.111	38	0.251
25a	Denmark, Lolland	54°47'30"	11°00'11"	0	7	1	2	0	10	0	11	3	4	0	18	46	0.199	34	0.196
25b	Denmark, Lolland	54°46'36"	11°00'34"	0	8	6	1	0	15	0	1	7	7	0	15	39	0.202	38	0.203
25c	Denmark, Lolland	54°45'17"	11°02'45"	0	12	3	0	0	15	0	6	10	9	0	25	58	0.189	49	0.213
26a	Denmark, Lolland	54°42'47"	11°41'43"	0	19	0	0	0	19	0	1	9	1	0	11	50	0.189	31	0.127
26b	Denmark, Lolland	54°42'55"	11°39'56"	0	2	8	2	0	12	0	0	16	3	0	19	33	0.192	36	0.075
138	Sweden, Scania	55°31'32"	12°55'45"	0	1	8	1	0	10	0	1	20	25	0	46	35	0.146	42	0.081
001	Sweden, Scania	55°35'17"	13°21'15"	2	23	4	1	0	30	1	8	10	10	0	29	54	0.150	39	0.119
011	Sweden, Scania	55°34'06"	13°19'47"	0	7	22	0	0	29	0	2	27	7	0	36	42	0.186	38	0.101
014	Sweden, Scania	55°34'08"	13°19'01"	0	16	11	0	0	27	0	9	15	5	0	29	49	0.177	33	0.192
032	Sweden, Scania	55°34'03"	13°12'53"	0	14	23	0	0	37	0	10	23	13	0	46	89	0.161	76	0.334
032A	Sweden, Scania	55°34'27"	13°13'03"	0	5	9	0	0	14	0	11	17	10	0	38	33	0.172	28	0.319
089	Sweden, Scania	55°36'34"	13°23'19"	0	4	5	9	0	18	0	2	10	33	0	45	39	0.173	61	0.101
102	Sweden, Scania	55°32'51"	13°17'13"	0	17	7	0	0	24	0	5	22	5	0	32	49	0.214	34	0.296
108	Sweden, Scania	55°33'09"	13°16'08"	0	8	12	1	0	21	0	4	18	7	0	29	28	0.167	25	0.248
111	Sweden, Scania	55°32'06"	13°12'33"	1	10	34	1	0	46	0	4	22	6	0	32	72	0.163	63	0.336
126	Sweden, Scania	55°33'59"	13°14'12"	0	6	10	0	0	16	0	6	13	22	0	41	36	0.178	40	0.299
134	Sweden, Scania	55°33'03"	13°21'22"	0	3	16	0	0	19	0	5	29	1	0	35	40	0.172	36	0.157

				Males > 55mm (LL>50)						Females > 55mm (LL>50)						Genetic samples			
Pond	Country	Latitude	Longitude	LL	LLR	LR	LRR	RR	n_m	LL	LLR	LR	LRR	RR	n_f	n_L	H_EL	n_R	H_ER
Pr	Germany Coast, continent	54°14'50"	10°11'25"	0	2	5	0	0	7	0	0	3	0	0	3	-	-	-	-
Fe11	Germany Coast, Fehmarn	54°31'16"	11°03'12"	0	8	0	0	0	8	0	2	14	0	0	16	-	-	-	-
Fe15	Germany Coast, Fehmarn	54°29'46"	11°08'37"	0	15	1	0	0	16	0	1	7	0	0	8	-	-	-	-
Fe16	Germany Coast, Fehmarn	54°29'45"	11°08'51"	0	14	0	0	0	14	0	1	8	0	0	9	-	-	-	-
Fe21	Germany Coast, Fehmarn	54°29'52"	11°08'45"	0	14	1	1	0	16	0	2	4	0	0	6	-	-	-	-
Fe70	Germany Coast, Fehmarn	54°25'35"	11°06'38"	0	13	2	0	0	15	0	7	8	0	0	15	-	-	-	-
Kl	Germany Coast, continent	53°59'29"	111°00'47"	0	7	2	2	0	11	0	1	9	6	0	16	-	-	-	-
Han7	Germany Coast, continent	54°02'27"	11°54'41"	0	13	0	0	0	13	0	3	1	0	0	4	-	-	-	-
Han10	Germany Coast, continent	54°02'44"	11°53'58"	0	9	2	0	0	11	0	5	2	0	0	7	-	-	-	-
Gr1	Germany Coast, continent	53°45'40"	12°55'49"	0	3	3	0	0	6	0	5	6	0	0	11	-	-	-	-
Gr11	Germany Coast, continent	53°41'36"	12°51'11"	0	7	0	3	0	10	0	1	0	3	0	4	-	-	-	-
Ru11	Germany Coast, Rügen	54°25'02"	13°23'49"	0	17	0	0	0	17	1*	1	2	0	0	4	-	-	-	-
Ru20	Germany Coast, Rügen	54°22'48"	13°24'52"	0	5	0	0	0	5	0	1	9	0	0	10	-	-	-	-
Ru43	Germany Coast, Rügen	54°21'25"	13°26'01"	0	3	2	7	0	12	0	0	6	6	0	12	-	-	-	-
Ru54	Germany Coast, Rügen	54°37'15"	13°22'21"	0	9	3	1	0	13	0	4	6	3	0	13	-	-	-	-
Ru74	Germany Coast, Rügen	54°16'33"	13°42'49"	0	27	0	0	0	27	0	6	2	0	0	8	-	-	-	-
UsZ	Germany Coast, Usedom	53°52'45"	14°07'48"	0	10	1	0	0	11	0	0	0	0	0	0	-	-	-	-
Us1	Germany Coast, Usedom	53°53'51"	14°03'55"	0	6	0	0	0	6	0	0	2	2	0	4	-	-	-	-
Ro4	Germany Coast, continent	53°34'26"	13°46'04"	1	5	0	0	0	6	0	8	3	3	0	14	-	-	-	-
Ro5	Germany Coast, continent	53°34'19"	13°45'38"	0	9	5	0	0	14	0	2	7	1	0	10	-	-	-	-
Do3	Germany Inland, continent	52°49'44"	09°16'18"	0	11	8	0	0	19	0	10	6	1	0	17	57	0.271	37	0.276
Do18	Germany Inland, continent	52°53'47"	09°16'26"	0	0	4	2	0	6	0	0	9	2	0	11	17	0.223	21	0.399
St	Germany Inland, continent	52°49'44"	09°21'27"	0	1	3	1	0	5	0	0	3	4	0	7	13	0.289	17	0.521
Hee	Germany Inland, continent	52°43'39"	09°17'02"	1	7	3	0	0	11	0	1	0	0	0	1	21	0.305	11	0.409
Ei	Germany Inland, continent	52°43'54"	09°36'45"	0	15	3	0	0	18	0	9	7	2	0	18	60	0.339	38	0.491
Sc	Germany Inland, continent	52°31'60"	09°19'47"	0	1	13	0	0	14	0	0	6	3	0	9	24	0.306	26	0.206
Eb	Germany Inland, continent	53°03'20"	10°27'14"	0	1	7	1	0	9	0	0	11	7	0	18	28	0.351	35	0.307
Da	Germany Inland, continent	53°29'17"	11°51'44"	0	1	2	11	0	14	0	0	1	6	0	7	22	0.281	38	0.303
Sh	Germany Inland, continent	52°54'05"	12°19'21"	0	4	4	2	0	10	0	2	4	4	0	10	26	0.314	26	0.393
Other breeding systems																			
Po	Germany Inland, continent	52°28'24"	09°28'28"	1	1	3	0	3	8	1	0	0	0	5	6	9	0.186	20	0.514
Ha2	Germany Inland, continent	52°15'23"	11°13'04"	0	0	3	2	0	5	0	0	0	3	1*	3	8	0.269	15	0.460
Ha6	Germany Inland, continent	52°16'40"	11°15'15"	4	2	3	0	0	9	1	1	3	1	0	6	23	0.313	11	0.468
Her	Germany Inland, continent	51°37'38"	10°21'14"	2	0	7	0	15	24	0	0	3	0	0	3	14	0.492	40	0.370
Bo3+4	Bornholm (Denmark)	55°08'39"	15°03'42"	0	0	10	3	3	16	0	0	0	0	6	6	-	-	-	-
Bo11	Bornholm (Denmark)	55°01'15"	15°01'15"	0	6	3	25	0	34	0	1	0	10	0	11	-	-	-	-
Bo12	Bornholm (Denmark)	55°03'25"	15°00'15"	0	3	0	14	2	19	0	0	0	0	0	0	-	-	-	-
Bo14	Bornholm (Denmark)	55°07'23"	15°09'10"	0	6	24	5	0	35	0	0	4	2	0	6	-	-	-	-
EE-NE	Baltic states, Estonia	59°05'14"	23°31'60"	19*	0	0	0	0	19							38	0.037	0	-
EE-L	Baltic states, Estonia	58°25'42"	26°19'08"	27*	0	0	0	0	27							-	-	-	-
EE-P	Baltic states, Estonia	58°21'50"	24°32'41"	35*	0	3*	0	0	38							-	-	-	-
EE-T	Baltic states, Estonia	58°08'09"	24°31'21"	41*	0	0	0	0	41							82	0.322	0	-
EE-H	Baltic states, Estonia	58°05'16"	24°29'39"	31*	0	5*	0	0	36							67	0.233	5	-
LV-P	Baltic states, Latvia	57°33'13"	24°44'52"	13*	0	9*	0	0	22		Sex and length					-	-	-	-
LV-A	Baltic states, Latvia	57°31'05"	24°56'38"	38*	0	0	0	0	38							76	0.364	0	-
LV-S	Baltic states, Latvia	57°19'41"	22°15'22"	27*	0	6*	0	0	33		not recorded					-	-	-	-
LV-R	Baltic states, Latvia	57°00'33"	23°10'54"	36*	0	4*	0	0	40							76	0.380	4	-
LV-J	Baltic states, Latvia	56°59'37"	23°55'22"	1*	0	0	0	14*	15		for Baltic frogs.					-	-	-	-
LV-B	Baltic states, Latvia	56°22'52"	24°10'06"	36*	0	5*	0	0	41							77	0.288	5	-
LT-G	Baltic states, Lithuania	55°19'04"	26°00'53"	16*	0	4*	0	0	20							-	-	-	-
LT-K	Baltic states, Lithuania	54°47'49"	24°15'05"	4*	0	3*	0	20*	27							11	0.374	43	0.615
LT-D	Baltic states, Lithuania	54°42'49"	24°05'54"	0	0	19*	0	10*	29							-	-	-	-
LT-BV	Baltic states, Lithuania	54°28'44"	25°07'59	16*	0	16*	0	1*	33							-	-	-	-

* Sex unknown

Curriculum Vitae

Personal

Name	Ditte Guldager Christiansen
Birth	19.03.1975 in Copenhagen, Denmark
Nationality	Danish
Civil status	Married to Daniel Leutwyler, Swiss

Education

2005-2009	PhD at the department of Ecology, Zoological Institute, University of Zurich, Switzerland. Thesis: Genetic structure and function of all-hybrid edible frog populations.
1995-2003	Bachelor's and Master's education in Biology at the University of Copenhagen, Denmark. Thesis at the departments of Evolution and Population Biology, Zoological Institute. Title: Genetic and reproduction in Danish pure populations of the hybrid water frog, *Rana esculenta*.
1991-1994	High school, mathematical line, at Kalundborg Gymnasium, Denmark.

International experience

2005-present	PhD in Zürich, Switzerland
2005-2007	7 months of PhD field work in Skåne, Sweden.
2000	2.5 months of volunteer work on a frigate bird project, Isla Isabel, Mexico.
1999	10 days of NGO work for Danish Nature and Youth in South Africa.
1997	2 months of volunteer work in two rainforest reserves in Ecuador.
1995-2004	Paid and voluntary work with firebellied toad conservation in Denmark
1994-1995	3 months of voluntary humanitarian work in Nicaragua.
1993-1994	40 lessons as Spanish Teacher at Kalundborg Evening School, Denmark
1991	6 months of college on Mallorca, Spain.
1989-2000	Camp, meeting and editor NGO activities in Nature and Youth, Denmark
1988-1989	11 months of school on the Faeroe Islands.

Languages Danish, English, Swiss German, German, Spanish, Swedish, Faroese.

Previous publications

Christiansen DG, K Fog, BV Pedersen, JJ Boomsma, 2005: Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. Evolution 59: 1348-1361.

Christiansen DG, 2005: A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. Molecular Ecology Notes 5: 190-193.